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Total Number of Pages in This Submission

Application Number	10/010,081
Filing Date	November 9, 2001
First Named Inventor	Didier
Art Unit	1633
Examiner Name	Kaushal, Sumesh
Attorney Docket Number	CLFR:010US

ENCLOSURES *(Check all that apply)*

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  - ☐ Fee Attached
- ☐ Amendment/Reply
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  - ☐ Affidavits/declaration(s)
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- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☑ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
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  Corrected Appeal Brief
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July 12, 2006 — Date

David L. Parker

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Didier Trono
Patrick Salmon

Serial No.: 10/010,081

Filed: November 9, 2001

For: METHODS AND COMPOSITIONS RELATING TO IMPROVED LENTIVIRAL VECTORS AND THEIR APPLICATIONS

Group Art Unit: 1636

Examiner: Kaushal, Sumesh

Atty. Dkt. No.: CLFR:010US/DLP

CORRECTED BRIEF ON APPEAL

MS Appeal Briefs
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Applicants hereby submit their Corrected Brief on Appeal pursuant to the Notice of Appeal filed February 10, 2006 and in response to the communication dated June 14, 2006. It is believed that no fees are due; however, if any such fees are due, the Commissioner is authorized to deduct any such fees from Fulbright & Jaworski LLP deposit account 50-1212/CLFR:010US.

(i) REAL PARTY IN INTEREST

The real party in interest is the Research Development Foundation.

(ii) RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

(iii) STATUS OF CLAIMS

Claims 1-3, 6, 26-29, and 35-37 are canceled. Claims 4, 5, 7-25, 30-34 and 38- 45 are pending, of which claims 11, 13-18, 20-21 and 24 are currently withdrawn. Pending claims 4-5, 7-10, 12, 19, 22, 23, 25, 30-34 and 38-45 have been finally rejected in an Office Action dated December 1, 2005, and are subject to the present appeal.

(iv) STATUS OF AMENDMENTS

No amendments have been sought subsequent to the close of prosecution.

(v) SUMMARY OF THE CLAIMED SUBJECT MATTER

The present case presents two independent claims, claims 30 and 32.

Claim 30 is directed to a human hematopoietic precursor cell that has been transduced with a self-inactivating recombinant lentivirus, also referred to as an "SIN" vector. Specification, page 4, lines 1-4; page 11, lines 4-9; page 7, lines 1-10. The lentivirus comprises an expression cassette that includes a transgene positioned under the control of a promoter that is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 10 and about 200 in both a human hematopoietic progenitor cell and a differentiated hematopoietic cell (Specification, pages 17, lines 23-28); and an LTR region that has reduced promoter activity relative to wild-type LTR. *Id.*; see also Specification, page 6, lines 1-12 (signal-to-noise ratio); pages 57-58 (expression in both progenitor and differentiated hematopoietic cells).

Of the pending claims under examination and subject to this appeal, claims 4-5, 7-10, 12-19, 22-25 and 30 depend from independent claim 30.

Claim 4 is directed to the transduced cell of claim 30, wherein the recombinant lentivirus is further defined as incapable of reconstituting a wild-type lentivirus through recombination. Specification, page 17, lines 13-16; page 21, lines 6-9, original claim 4.

Claim 5 is directed to the transduced cell of claim 4, wherein the recombinant lentivirus does not express a functional lentiviral gene. Specification, page 5, lines 8-15.

Claim 7 is directed to the transduced cell of claim 30, wherein the promoter is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 40 and about 200. Specification, page 6, lines 1-12.

Claim 8 is directed to the transduced cell of claim 7, wherein the promoter is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 150 and about 200. Specification, page 6, lines 1-12.

Claim 9 is directed to the transduced cell of claim 30, wherein the promoter is an EF1-α promoter, a PGK promoter, a gp91phox promoter, a MHC classII promoter, a clotting Factor IX promoter, a clotting Factor V111 promoter, an insulin promoter, a PDX1 promoter, a CD11 promoter, a CD4 promoter, a CD2 promoter or a gp47 promoter, with claim 10 specifying the EF1-α promoter. Specification, page 6, lines 14-19.

Claim 12 is directed to the transduced cell of claim 30, wherein the transgene is erythropoietin, an interleukin, a colony-stimulating factor, integrin αIIbβ, a multidrug resistance gene, gp91phox, gp 47, an antiviral gene, a gene coding for blood coagulation factor VIII, a gene coding for blood coagulation factor IX, a T cell antigen receptor, a B cell antigen receptor, a

single chain antibodies (ScFv), TNF, gamma interferon, CTLA4, B7, Melana, MAGE; with many of these species being broken out in dependent claims 13-18 . Specification, page 8, line 20 to page 9, line 6.

Claim 19 is directed to the transduced cell of claim 30, further comprising a posttranscriptional regulatory sequence positioned to promote the expression of the transgene, such as a posttranscriptional regulatory element (claim 22), such as the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) (claim 23). Specification, page 8, lines 9-17.

Claim 25 is directed to the transduced cell of claim 30, wherein the LTR region has been rendered substantially transcriptionally inactive by virtue of deletions in the U3 region of the 3' LTR. Specification, page 7, lines 13-28.

Lastly, claim 31 is directed to the transduced host cell of claim 30, wherein the human hematopoietic progenitor cell is a $CD34^+$ cell. Specification, page 11, lines 4-9.

Independent claim 32 concerns a method for transducing a human hematopoietic stem cell comprising contacting a population of human cells that include hematopoietic stem cells *in vitro* with a lentiviral vector under conditions to effect the transduction of a human hematopoietic progenitor cell in said population by said vector. Specification, page 11, lines 11-22. The lentiviral vector is defined as a self-inactivating recombinant vector comprising an expression cassette comprising a transgene positioned under the control of a promoter that is that is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 10 and about 200 in a differentiated hematopoietic cell active to promote detectable transcription of the transgene in a human hematopoietic progenitor cell and an LTR region that has reduced promoter activity relative to wild-type LTR. (See above regarding claim 30) The final step of

the method of claim 32 comprises differentiating the transduced stem cell into a differentiated hematopoietic cell. Specification, page 17, lines 21-29; pages 57-58.

Claims 33, 34 and 38-45 depend from claim 32.

Dependent claim 33 is directed to the method of claim 32, wherein the human hematopoietic stem cell population comprises $CD34^+$ cells. Specification, page 12, lines 9-13.

Claim 34 is directed to the method of claim 32, wherein the cell population is treated to stimulate cell proliferation without substantial loss of stem cell pluripotency. See, original claim 34.

Claim 38 is directed to the method of claim 32, wherein the transduced stem cell is incubated in a differentiation media. Specification, pages 57-58.

Claim 39 is directed to the method of claim 38, wherein incubated transduced stem cell is differentiated into an erythroid cell, a granulocyte, a monocyte or a dendritic cell, and claims 40-45 break these out into individual cell types. Specification, pages 17, lines 23-28; pages 57-58.

(vi) ISSUES TO BE REVIEWED ON APPEAL

A. Whether the subject matter of claims 4-5, 7-8, 12, 25, 30-34 and 38-45 is obvious over the combination of Zufferey *et al.*, *J. Virol.*, 1998 (exhibit 1, evidence appendix ix; hereinafter "Zufferey I") in view of Deisseroth (exhibit 2, evidence appendix ix);

B. Whether the subject matter of claims 7-10 is obvious over the combination of Zufferey I and Deisseroth, as above, further in view of Chang *et al.* (exhibit 3, evidence appendix ix; hereinafter "Chang"); and

C. Whether the subject matter of claims 19, 22 and 23 is obvious over the combination of Zufferey I and Deisseroth, as above, further in view of Zufferey *et al.*, *J. Virol.*, 1999 (exhibit 4, evidence appendix ix; hereinafter "Zufferey II").

Appellants note that there is additionally a provisional double patenting rejection over copending application serial number 10/261,078. In that the '078 case is in active prosecution, with the claims having been recently amended but not presently allowed. In that the rejection is provisional, Appellants will address that rejection should the '078 case be allowed, and should the allowed claims give rise to a proper double-patenting rejection, by filing a terminal disclaimer.

(vii) ARGUMENT

A. Whether the subject matter of claims 4-5, 7-8, 12, 25, 30-34 and 38-45 is obvious over the combination of Zufferey I in view of Deisseroth.

1. The Rejection

The Action rejects claims 4-5, 7-8, 12, 25, 30-34 and 38-45 as obvious over Zufferey I in view of Deisseroth, taking the position that Zufferey I teaches a self-inactivating lentivirus vector ("SIN") comprising a transgene under the control of a CMV promoter and teaches its use in effectively transducing peripheral blood lymphocytes ("PBLs"; *i.e.*, *mature* hematopoietic cells). The Action states that the CMV promoter is inherently known to promote detectable transcription in human hematopoietic *progenitor* cells, referring to Case *et al.*, 1999 (exhibit 5, evidence appendix ix; hereinafter "Case"). The Action continues by alleging that the art teaches that "inactivation of LTR provides higher signal-to-noise ratio which falls in the range of about 10 to about 200" referring to page 9876, table 2, of Zufferey I. The Action further relies on figure 4, page 9878, for the proposition that Zufferey I teaches that inactivation of the

endogenous LTR promoter in combination with the use of an exogenous promoter demonstrates a transgene expression within the claimed signal-to-noise ratio of about 10 to about 200.

The Action concedes that Zufferey I does not teach transduction of hematopoietic progenitor cells as required by the claims, but takes the position that such a suggestion is supplied by Deisseroth, which is said to teach clinical trials involving the use of lentiviral vectors to transduce hematopoietic progenitor cells (such as CD34+ cells).

2. Appellants Argument

Claims 30, 32 and Claims Depending Therefrom

In response, the present invention, as represented by claims 30 and 32, is directed to human hematopoietic stem cells (or the preparation of same) transduced with an self-inactivating recombinant lentivirus expressing a transgene under control of a promoter that is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 10 and about 200 in ***both*** human hematopoietic progenitor cells ***and*** a differentiated hematopoietic cell. This is an important aspect in that many therapeutic applications of such lentiviruses require the ability of a particular lentivirus to express the transgene efficaciously in both such cell types – *i.e.*, both upon transduction of the initial progenitor cell and when the progenitor cell differentiates into a differentiated or mature hematopoietic cell. For the reasons set forth below, the Action fails to set forth a *prima facie* case of obviousness.

The prior art has traditionally employed exogenous promoters such as the CMV promoter (as opposed to the internal lentiviral LTR promoter) in the preparation of recombinant lentiviruses. Indeed, it is evident that many such promoters are capable of effecting some level of expression of lentiviral transgenes in *some, mature* hematopoietic cells. This is shown in the excerpts of Zufferey I relied on by the Examiner, including page 9876, table 2, and figure 4, page

9878.[1] However, all of these teachings are limited to transgene expression in mature hematopoietic cells and says nothing about lentiviral transgene expression in hematopoietic progenitor cells ("HPCs").

It is submitted that the present invention is directed to the inventors' discovery that certain promoters, such as the CMV promoter, express only poorly, if at all, in HPCs:

> While lentiviral vectors offer a great potential for gene-therapy and especially the transduction of human hematopoietic stem cells (hHSC), vectors developed so far have failed to meet biosaftey standards and are still inefficient in expression of transgenes. For example, while ***CMV promoter-containing HIV-derived vectors*** can induce high levels of transgene expression in the central nervous system (Naldini *et al.*, 1996a; Naldini *et al.*, 1996b; Blomer *et al.*, 1997), and allowed the initial demonstration that pluripotent hematopoietic precursors can be efficiently transduced by this gene delivery tool, ***they are largely useless for transferring therapeutic genes into most lympho-hematopoietic cells, because in these targets their transcriptional activity is prohibitively low*** (Miyoshi *et al.*, 1999; Case *et al.*, 1999; An *et al.*, 2000). Current lentiviral vectors have multiply attenuated HIV virulence genes which removes the potential for reconstitution of wild-type virus by recombination (Zufferey *et al.*, 1997; Dull *et al.*, 1998). A self-inactivating design rendered the vectors further biologically safe by eliminating the transcriptional elements of HIV (Zufferey *et al.*, 1998). However, this can negatively affect transgene expression, apparently by decreasing the efficiency of polyadenylation (DeZazzo *et al.*, 1991; Valsamakis *et al.*, 1991; Brown *et al.*, 1991; Cherrington and Ganem, 1992; Valsamakis *et al.*, 1992; Gilmartin *et al.*, 1992).

Specification, page 17, lines 3-19 (emphasis supplied). The specification proceeds by noting:

> However, CMV promoters are not preferred. For example, the inventors demonstrate using the green fluorescent protein (GFP) expression that while the EF1α promoter element or the PGK promoter were highly active in $CD34^+$ cells as well as several other differentiated hematopoietic derivatives, the CMV promoter driven GFP production was insufficient to determine either the percentage of transduced cells or the level of transgene expression in those cells.

Specification, page 18, lines 3-8.

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[1] Actually, only figure 4 of Zufferey I presents a study that enables one to assess a signal-to-noise ratio. In the figure 4 study, a signal-to-noise ratio of greater than 10 is achieved in the case of "Full-Length" and "SIN-78" constructs. However, all of the Zufferey I figure 4 studies concerned differentiated human lymphocytic SupT1 cells, ***not*** hematopoietic progenitor cells.

Indeed, Appellants' specification proceeds to provide studies demonstrating that the CMV promoter is unfit for use in hematopoeitic progenitor cells. The Board is referred to the studies shown in Figure 1 and described at page 55, line 13, through page 56, line 2:

> As shown in FIG. 1A, a sharp subpopulation of GFP-positive hematopoietic progenitors could only be seen when cells were transduced with HIV vectors containing the PGK or the EF1α promoters. Cells transduced with MLV vector or HIV vector containing the CMV promoter displayed only a small percentage of GFP-positive cells, together with a high heterogeneity in GFP expression. A side-by-side comparison of GFP expression after transduction of HeLa and $CD34^+$ cells revealed that the promoters examined behaved quite differently in these two cell types. The PGK promoter was weak in HeLa cells (overlap between GFP^+ and GFP^- cells) and strong in HPCs. The EF1α promoter was intermediate in HeLa cells and very potent in HPCs, with a mean value typically 100 times higher in transduced than in control HPCs. The CMV promoter, whether in an MLV or an HIV vector, was equally strong in HeLa cells, contrasting with its low activity in transduced HPCs. A PCR-based quantification performed at the time of the flow cytometry analysis (FIG. 1B) indicated that the low expression from the MLV-CMV vector in HPCs was due at least in part to poor gene transfer in these cells, a consequence of the limited stimulation of $CD34^+$ cells during transduction. In contrast, the low expression in HPCs exposed to the HIV-CMV vector (FIG. 1A) could not be explained by a low frequency of transduction, since equivalent amounts of transgene DNA were present in cells transduced with all three HIV vectors (FIG. 1B). This indicates that the CMV promoter does not govern efficient expression in hematopoietic progenitors, a finding in accordance with previous reports (Miyoshi *et al.*, 1999; Case *et al.*, 1999; An *et al.*, 2000).

Specification, page 55, line 13, through page 56, line 2. Indeed, referring to Figure 1A (Exhibit 6; evidence appendix), it is seen that HPC/CD34+ cells transduced with a lentiviral/CMV construct was found by the inventors to result in virtually undetectable expression of GFP, as contrasted with other promoters, which showed generally high level expression in the HPCs.

Accordingly, through the inclusion of the "signal-to-noise" ratio limitation, the claims have been drafted to exclude the use of the CMV promoter, since, as shown by Appellants' studies, it is incapable of effecting the claimed signal-to-noise ratio in the context of lentiviral SIN vector in both HPCs and in differentiated hematopoietic cells.

Returning to the rejection, it is submitted that the Examiner has failed to make a *prima facie* showing of obviousness. Zufferey I fails to teach of suggest ***not*** to use the CMV promoter in the context of lentiviral SIN vectors, and Deisseroth fails to remedy this deficiency. Accordingly, one of skill faced with the combination of Zufferey I and Deisseroth would be directed to use a CMV promoter, which, as shown by Appellants' studies, would not result in achieving the subject matter of either of claim 30 or 32 or claims depending therefrom.

Claim 32, in particular, and Claims Depending Therefrom

Turning additionally to claim 32 and dependents therefrom, we would first note that this claim is similarly directed to the use of a promoter that is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 10 and about 200 in a differentiated hematopoietic cell, and thus for the reasons discussed above, exclude the use of the CMV promoter. However, claim 32 also includes the further step of differentiating the transduced stem cell into a differentiated hematopoietic cell.

With respect to the inclusion in claim 32 of the further step of differentiating the transduced stem cell into a differentiated hematopoietic cell, we have been unable to identify any teaching or suggestion of such a step in either of the cited references. In the final office action, the Examiner merely refers us to "Deisseroth" without referencing an excerpt. We have reviewed Deisseroth and can only find one section that arguably appears to relate to transduction of HSCs/CD34+ cells with lentivirus (or any other virus), section (d) on page 1608, column 2. However, we have been unable to identify any teaching here relating to "differentiating the transduced stem cell into a differentiated hematopoietic cell." If we have overlooked the passage being relied upon by the Examiner, he is respectfully requested to identify it in his Answer so that we might properly consider and address the argument.

In any event, in addition to the arguments presented above with respect to all of the rejected claims, Appellants present this additional argument with respect to claim 32 and its dependents.

Claim 38

Claim 38 specifies incubation of the transduced stem cell in a differentiation medium. Appellants have been unable to identify any rejection applicable to this claim and submits that it is still further removed from the teachings of the art of record in that Applicants have been unable to identify therein any applicable teaching or suggestion. If we have overlooked the passage being relied upon by the Examiner, he is respectfully requested to identify it in his Answer so that we might properly consider and address the argument.

Claims 39-45

Appellants submit that the subject matter of each of claims 39-45 are separately patentable and further removed from the prior art of record. In particular, claim 39 is directed to incubating the transduced stem cell to differentiate it into an erythroid cell, a granulocyte, a monocyte or a dendritic cell. Claims 40 – 45 are dependent claims directed specifically to producing a dendritic cell (claim 40), a granulocyte (claim 41), an erythroid cell (claim 42), a monocyte (claim 43), a B cell (claim 44) or a T lymphocyte (claim 45). These claims are also submitted to be, individually, separately patentable over the art.

Again, Appellants have been unable to identify any rejection applicable to this claim and submits that it is still further removed from the teachings of the art of record in that Applicants have been unable to identify therein any applicable teaching or suggestion. If we have overlooked the passage being relied upon by the Examiner, he is respectfully requested to identify it in his Answer so that we might properly consider and address the argument.

B. Whether the subject matter of claims 7-10 is obvious over the combination of Zufferey I and Deisseroth, as above, further in view of Chang et al. (exhibit 3, appendix ix; hereinafter "Chang").

The Action next rejects claims 6-10 as obvious over the same combination, further in view of Chang, which is said to teach lentiviral vectors incorporating an EF1-α promoter.

In response, Appellants first incorporate by reference the arguments set forth above with respect to claim 30.

Furthermore, it is our position that there is no motivation to combine the teachings of Chang with those of Zufferey I, in that there was no reasonable expectation that the SIN design would work in hematopoeitic cells. We have been unable to identify any teaching *per se* in Zufferey I that would suggest to employ the SIN design in the context of hematopoietic progenitor cells. If the Examiner is aware of any such teaching she is respectfully requested to point it out. In fact, the SIN design incorporates modifications in their LTR region that reduces their promoter activity, and there was simply no way of knowing in advance what effect this would have on its ability to transfect and express in such cells. The reason for this is that neither the transcriptional milieu nor the specificities in hematopoietic cells, particularly hematopoietic progenitor cells, have been well characterized. As a consequence, the behavior of internal promoters with respect to the LTR regions in the context of a SIN design could not be predicted in advance. Thus, without having a reasonable expectation that a SIN vector could be successfully employed in hematopoietic cells, there would be no reason or basis for modifying the SIN-CMV construct of Zufferey I. Futhermore, Zufferey I not only fails to suggest the applicability of SIN design vectors to hematopoietic progenitor cells, it also appears to be silent as to any drawbacks associated with the CMV promoter in this or any context.

The Action apparently attempts to confront the foregoing argument by taking the position that Chang demonstrates that usefulness of the EF1-α promoter in a lentivector in the context of CD34+ cells. However, this is not precisely the case – the lentivector employed by Chang was NOT a SIN design, which as explained above can have a substantial effect on promoter behaviour and transgene expression, and thus there is no way to predict in advance of the present application that promoters taught be Chang could be used advantageously in the context of the SIN design. Additionally, Chang fails to demonstrate whether the EF1-α promoter would remain active upon differentiation in differentiated hematopoietic cells, which would not have been clear to one of skill in the art at the time the invention was made. In contrast, the inventors demonstrate that when hematopoietic progenitor cells are transduced by vectors comprising the EF1-α promoter and then differentiated "transgene expression was high in all lineages examined after transduction" (see page 57 lines 23-29 of the specification). This result would clearly ***not*** have been obvious to one of skill in the art, since as pointed out in the specification, other promoters (*e.g.* CMV) are "only minimally active in most progenitor cells" (page 6 lines 17 – 19). Thus, at the time of invention it would have been unclear to one of skill in the art that there was any advantage to using the EF1-α promoter, especially in the context of a SIN vector.

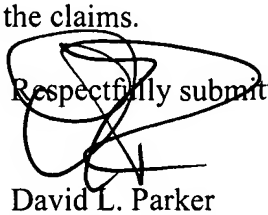
Accordingly, a *prima facie* obviousness rejection has not been properly set forth.

C. *Whether the subject matter of claims 19, 22 and 23 is obvious over the combination of Zufferey I and Deisseroth, as above, further in view of Zufferey et al., J. Virol., 1999 (exhibit 4, appendix ix; hereinafter "Zufferey II").*

In response, with respect to this rejection, Appellants incorporate by reference the arguments set forth above with respect to claim 30, from which Claims 19, 22 and 23 depend.

Conclusion

It is submitted that by the foregoing submissions and arguments, Appellants request that the Board overturn the Examiner's rejection of the claims.



Respectfully submitted,

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Date: July 12, 2006

(viii) CLAIMS APPENDIX

1. – 3. (Canceled)

4. (Previously presented) The transduced cell of claim 30, wherein the recombinant lentivirus is further defined as incapable of reconstituting a wild-type lentivirus through recombination.

5. (Previously presented) The transduced cell of claim 4, wherein the recombinant lentivirus does not express a functional lentiviral gene.

6. (Canceled)

7. (Previously presented) The transduced cell of claim 30, wherein the promoter is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 40 and about 200.

8. (Previously presented) The transduced cell of claim 7, wherein the promoter is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 150 and about 200.

9. (Previously presented) The transduced cell of claim 30, wherein the promoter is an EF1-α promoter, a PGK promoter, a gp91phox promoter, a MHC classII promoter, a clotting Factor IX promoter, a clotting Factor V111 promoter, an insulin promoter, a PDX1 promoter, a CD11 promoter, a CD4 promoter, a CD2 promoter or a gp47 promoter.

10. (Previously presented) The transduced cell of claim 9, wherein the transgene is positioned under the control of the EF1-α promoter.

11. (Withdrawn) The vector of claim 9, wherein the transgene is positioned under the control of the PGK promoter.

12. (Previously presented) The transduced cell of claim 30, wherein the transgene is erythropoietin, an interleukin, a colony-stimulating factor, integrin αIIbβ, a multidrug resistance gene, gp91phox, gp 47, an antiviral gene, a gene coding for blood coagulation factor VIII, a gene coding for blood coagulation factor IX, a T cell antigen receptor, a B cell antigen receptor, a single chain antibodies (ScFv), TNF, gamma interferon, CTLA4, B7, Melana, MAGE.

13. (Withdrawn) The transduced cell of claim 12, wherein the transgene is gp91phox.

14. (Withdrawn) The transduced cell of claim 12, wherein the transgene is gp 47.

15. (Withdrawn) The transduced cell of claim 12, wherein the transgene is Interleukin-2.

16. (Withdrawn) The transduced cell of claim 12, wherein the transgene is Interleukin-12.

17. (Withdrawn) The transduced cell of claim 12, wherein the transgene is a gene coding for blood coagulation factor VIII.

18. (Withdrawn) The transduced cell of claim 12, wherein the transgene is a gene coding for blood coagulation factor IX.

19. (Previously presented) The transduced cell of claim 30, further comprising a posttranscriptional regulatory sequence positioned to promote the expression of the transgene.

20. (Withdrawn) The vector of claim 19, wherein the posttranscriptional regulatory sequence is an intron positioned within the expression cassette.

21. (Withdrawn) The vector of claim 20, wherein the intron is positioned in an orientation opposite the vector genomic transcript.

22. (Previously presented) The transduced cell of claim 19, wherein the posttranscriptional regulatory sequence is a posttranscriptional regulatory element.

23. (Previously presented) The transduced cell of claim 22, wherein the posttranscriptional regulatory element is woodchuck hepatitis virus posttranscriptional regulatory element (WPRE).

24. (Withdrawn) The vector of claim 23, wherein the posttranscriptional regulatory element is hepatitis B virus posttranscriptional regulatory element (HPRE).

25. (Previously presented) The transduced cell of claim 30, wherein the LTR region has been rendered substantially transcriptionally inactive by virtue of deletions in the U3 region of the 3' LTR.

26.-29. (Canceled)

30. (Previously presented) A human hematopoietic cell transduced with a self-inactivating recombinant lentivirus, the lentivirus comprising an expression cassette comprising a transgene positioned under the control of a promoter that is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 10 and about 200 in both a human hematopoietic progenitor cell and a differentiated hematopoietic cell; and an LTR region that has reduced promoter activity relative to wild-type LTR, wherein the human hematopoietic cell is a human hematopoietic progenitor cell.

31. (Previously presented) The transduced host cell of claim 30, wherein the human hematopoietic progenitor cell is a $CD34^+$ cell.

32. (Previously presented) A method for transducing a human hematopoietic stem cell comprising the steps of:

(i) contacting a population of human cells that include hematopoietic stem cells *in vitro* with a lentiviral vector under conditions to effect the transduction of a human hematopoietic

progenitor cell in said population by said vector, wherein the lentiviral vector is defined as a self-inactivating recombinant vector comprising:

(a) an expression cassette comprising a transgene positioned under the control of a promoter that is that is capable of promoting expression of the transgene at a signal-to-noise ratio of between about 10 and about 200 in a differentiated hematopoietic cell active to promote detectable transcription of the transgene in a human hematopoietic progenitor cell; and

(b) an LTR region that has reduced promoter activity relative to wild-type LTR; and

(ii) differentiating the transduced stem cell into a differentiated hematopoietic cell.

33. (Original) The method of claim 32, wherein the human hematopoietic stem cell population comprises $CD34^+$ cells.

34. (Original) The method of claim 32, wherein the cell population is treated to stimulate cell proliferation without substantial loss of stem cell pluripotency.

35. – 37. (Canceled)

38. (Previously presented) The method of claim 32, wherein the transduced stem cell is incubated in a differentiation media.

39. (Previously presented) The method of claim 38, wherein incubated transduced stem cell is differentiated into an erythroid cell, a granulocyte, a monocyte or a dendritic cell.

40. (Previously presented) The method of claim 39, wherein the incubated transduced stem cell is differentiated into a dendritic cell.

41. (Previously presented) The method of claim 39, wherein the incubated transduced stem cell is differentiated into a granulocyte.

42. (Previously presented) The method of claim 39, wherein the incubated transduced stem cell is differentiated into an erythroid cell.

43. (Previously presented) The method of claim 39, wherein the incubated transduced stem cell is differentiated into a monocyte.

44. (Previously presented) The method of claim 39, wherein the incubated transduced stem cell is differentiated into a B cell.

45. (Previously presented) The method of claim 39, wherein the incubated transduced stem cell is differentiated into a T lymphocyte.

(ix) **EVIDENCE APPENDIX**

Exhibit 1 – Zufferey *et al.*, *Journal of Virology, 72*:9873-9880 (1998); Office Action dated June 18, 2004, page 6.

Exhibit 2 – Deisseroth, *Clinical Cancer Research, 5*:1607-1609 (1999); Office Action dated June 18, 2004, page 7.

Exhibit 3 – Chang *et al., Gene Therapy 6*:715-728 (1999); Office Action dated June 18, 2004, page 8.

Exhibit 4 – Zufferey *et al., Journal of Virology, 73*:2886-2892 (1999); Office Action dated June 18, page 10.

Exhibit 5 – Case *et al., Proc. Natl. Acad. Sci, USA, 96*-:2988-2993 (1999): Office Action dated December 1, 2005, page 4, line 4.

Exhibit 6 – Figure 1A of Applicants' patent specification, filed November 9, 2001.

Exhibit 1

Zufferey *et al.*, *Journal of Virology, 72*:9873-9880 (1998); Office Action dated June 18, 2004, page 6.

JOURNAL OF VIROLOGY, Dec. 1998, p. 9873–9880
0022-538X/98/$04.00+0
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Vol. 72, No. 12

Self-Inactivating Lentivirus Vector for Safe and Efficient In Vivo Gene Delivery

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Received 1 June 1998/Accepted 13 August 1998

In vivo transduction of nondividing cells by human immunodeficiency virus type 1 (HIV-1)-based vectors results in transgene expression that is stable over several months. However, the use of HIV-1 vectors raises concerns about their safety. Here we describe a self-inactivating HIV-1 vector with a 400-nucleotide deletion in the 3′ long terminal repeat (LTR). The deletion, which includes the TATA box, abolished the LTR promoter activity but did not affect vector titers or transgene expression in vitro. The self-inactivating vector transduced neurons in vivo as efficiently as a vector with full-length LTRs. The inactivation design achieved in this work improves significantly the biosafety of HIV-derived vectors, as it reduces the likelihood that replication-competent retroviruses will originate in the vector producer and target cells, and hampers recombination with wild-type HIV in an infected host. Moreover, it improves the potential performance of the vector by removing LTR sequences previously associated with transcriptional interference and suppression in vivo and by allowing the construction of more-stringent tissue-specific or regulatable vectors.

Retroviral vectors are attractive tools for human gene therapy. First, they stably integrate into the chromosomes of their targets, a likely requisite for long-term expression. Second, they do not transfer viral genes, avoiding transduced cells that are destroyed by virus-specific cytotoxic T cells. Third, they have a relatively large cloning capacity, sufficient for most envisioned clinical situations. In addition to these characteristics, which are common to all retroviral vectors, vectors derived from lentiviruses offer one great advantage over their oncoretroviral counterparts: they can transduce nondividing cells, a crucial asset for genetically modifying tissues considered the main potential targets of gene therapy, such as the brain, the muscle, the liver, the lungs, and the hematopoietic system. Illustrating these properties, vectors derived from human immunodeficiency virus type 1 (HIV-1) allow for the efficient in vivo delivery, integration, and stable expression of transgenes into cells such as neurons, hepatocytes, and myocytes (2, 14, 17, 18). Although this opens exciting prospects for human gene therapy, the biosafety of HIV-based vectors requires a most careful evaluation, considering the pathogenicity of the parental virus.

Two components are involved in the making of a virus-based gene delivery system: first, the packaging elements, encompassing the structural proteins as well as the enzymes necessary to generate an infectious particle, and second, the vector itself, that is, the genetic material which will be transferred to the target cell. Biosafety safeguards, one goal of which is to prevent the emergence of replication-competent recombinants (RCRs), can be introduced in designing both of these components.

The packaging unit of the first generation of HIV-based vectors comprised all of the HIV-1 proteins except the envelope (18). A major step towards clinical acceptability was the subsequent demonstration that the fundamental properties of this system were left intact after deletion of four additional viral genes, encoding proteins proven or likely to represent crucial virulence factors: Vpr, Vif, Vpu, and Nef (31). More recent studies now indicate that the main transactivator of HIV, Tat, is also dispensable for generation of a fully efficient vector (7). What could be termed the third-generation packaging unit of HIV-1-based vectors thus conserves only three of the nine genes present in the genome of the parental virus: *gag*, *pol*, and *rev*. This eliminates the possibility that a wild-type virus will be reconstituted through recombination.

The system would be further improved if the transcriptional elements of HIV were removed from the vector. The modalities of reverse transcription, which generates both U3 regions of an integrated provirus from the 3′ end of the viral genome, facilitate this task by allowing the creation of so-called self-inactivating (SIN) vectors. Self-inactivation relies on the introduction of a deletion in the U3 region of the 3′ long terminal repeat (LTR) of the DNA used to produce the vector RNA. During reverse transcription, this deletion is transferred to the 5′ LTR of the proviral DNA. If enough sequence is eliminated to abolish the transcriptional activity of the LTR, the production of full-length vector RNA in transduced cells is abolished. This minimizes the risk that RCRs will emerge. Furthermore, it reduces the likelihood that cellular coding sequences located adjacent to the vector integration site will be aberrantly expressed, either due to the promoter activity of the 3′ LTR or through an enhancer effect. Finally, a potential transcriptional interference between the LTR and the internal promoter driving the transgene is prevented by the SIN design.

SIN vectors have been derived from murine leukemia virus (MLV) and spleen necrosis virus (SNV) (6, 12, 29, 30). Their development, however, has highlighted some of the difficulties inherent in this approach. The 3′ LTR is indeed involved in the polyadenylation of the viral RNA, a function that requires sequence elements often spread over U3, R, and U5. A U3 deletion conferring self-inactivation must eliminate as many of the transcriptionally important motifs from the LTR as possible while sparing the polyadenylation determinants. Because of overlaps between these two functional entities, most MLV-

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derived SIN vectors carry a deletion limited to the enhancer and as a consequence conserve significant transcriptional activity in their LTRs. One attempt to mutate the TATA box dramatically decreased the titers of the resulting vector, presumably because polyadenylation was rendered inefficient (29).

Studies on the regulation of HIV-1 polyadenylation have located the main *cis*-acting element governing the polyadenylation of the viral genomic RNA distal to the TATA box, just upstream of the R region of the LTR (5, 26, 27). This suggests that HIV-1-derived vectors may tolerate large U3 deletions and even a complete removal of the viral promoter without functional loss. Verifying this prediction, we report here on the successful development of HIV-based SIN vectors. Extensive U3 deletions, including one which removed the TATA box and resulted in an almost complete loss of LTR promoter activity, could be introduced without altering vector titers. Furthermore, none of the in vitro and in vivo properties of HIV-derived vectors were compromised by the SIN configuration.

MATERIALS AND METHODS

SIN plasmids. (i) pHR'SIN plasmids. A *Kpn*I-*Xba*I fragment containing the polypurine tract and the 3' LTR was excised from a pHR' plasmid and subcloned into the corresponding sites of pUC18. This plasmid was digested completely with *Eco*RV and partially with *Pvu*II and self-ligated. A plasmid carrying a 400-nucleotide-long deletion of U3 was recovered. An *Xho*I linker was inserted in the *Eco*RI site of the deletion plasmid, and an *Xho*I-*Xba*I fragment was cloned back into the pHR'CMVlacZ plasmid digested with the corresponding enzymes. All other SIN-18 plasmids were obtained by substituting reporter genes (encoding luciferase, enhanced green fluorescence protein [GFP], and Neo) for *lacZ*. All reporter genes were swapped as *Bam*HI-*Xho*I cassettes. The pHR' vector plasmids used in this study differ from the plasmids originally described (17) by an *Xho*I-*Kpn*I deletion removing 118 nucleotides from the Nef-coding sequence upstream of the polypurine tract and a deletion of 1,456 nucleotides of human sequence downstream of the 3' LTR. This human sequence remained from the original cloning of the HXB2 proviral genome. The two deletions did not affect vector titers or transgene expression in dividing 293T cells.

(ii) pRLLSIN plasmids. The construction of pRRL plasmids containing a chimeric 5' LTR made of Rous sarcoma virus U3 and HIV-1 R/U5 regions is described elsewhere (7). pRRLPGK-GFPSIN-18, pRRLPGK-GFPSIN-36, pRRLPGK-GFPSIN-45, and pRRLPGK-GFPSIN-78 are vectors in which the 3' LTR sequences from position −418 to −18, −36, −45, and −78, respectively, have been deleted from pRRLPGK-GFP.

pRRLPGK-GFPSIN-18 was generated by replacing the 590-bp *Eco*RI-*Afl*II fragment from pRRLPGK-GFP with the 200-bp *Eco*RI-*Afl*II fragment from pHR'CMVlacZSIN-18 in a four-part ligation with a 2.95-kb *Afl*II fragment, a 2.8-kb *Afl*II-*Bam*HI fragment, and a 760-bp *Bam*HI-*Eco*RI fragment from pRRLPGK-GFP.

pRRLPGK-GFPSIN-36 was derived from pRRLPGK-GFP by replacing the 493-bp *Bbs*I-*Alw*NI fragment in the 3' LTR with an oligonucleotide linker consisting of 5'-GATATGATCAGATC-3' and 5'-CTGATCA-3'. The linker was ligated with a 540-bp *Alw*NI-*Avr*II fragment and a 6.1-kb *Avr*II-*Bbs*I fragment from pRRLPGKGFP in a three-part ligation. pRRLPGK-GFPSIN-45 was generated similarly by using the oligonucleotides 5'-GATATGATCAGAGCCCTCAGATC-3' and 5'-CTGAGGGCTCTGATCA-3'. The two oligonucleotides 5'-GATATGATCAGGAGGCGTGGCCTGGGCGGGACTGGGGAGTGGCGAGCCCTCAGATC-3' and 5'-CTGAGGGCTCGCCACTCCCCAGTCCCGCCCAGGCCACGCCTCCTGATCA-3' were used to generate pRRLPGK-GFP-SIN-78.

Other plasmids. The envelope plasmid pMD.G and the packaging plasmid pCMVΔR8.91 have been described previously (31).

Cells. Dulbecco's modified Eagle medium (Gibco) was supplemented with 10% fetal calf serum and a combination of penicillin-streptomycin and glutamine (Gibco). 293T, HeLa, HeLa-tat, 208 F, and NIH 3T3 cells were cultured in supplemented Dulbecco's modified Eagle medium in a 10% CO_2 atmosphere. SupT1 cells were cultured in RPMI 1640 medium (modified) (JRH Biosciences) supplemented with 10% fetal calf serum and 2 mM L-glutamine in a 5% CO_2 atmosphere. Rat thyroid PC C13 cell lines immortalized with either E1A or v-Raf have been described previously (1). Primary human T lymphocytes were isolated and transduced as previously described (8). Gamma irradiation (8,000 rads) was delivered to cells in suspension as in previous studies (31) by a 3-min exposure to a ^{60}Co source.

Northern blot analysis. Total RNA was isolated from transduced HeLa cells by using RNAsol B as suggested by the manufacturer. RNA (10 to 20 μg) was separated on 1% agarose gel by using NorthernMax (Ambion) reagents and transferred to a Zetabind membrane by capillary transfer. A GFP-specific probe was ^{32}P labelled by random priming.

Vector stock preparation. Stocks were prepared as previously described (31) by transient cotransfection of three plasmids into 293T cells. The p24 concentration was determined by antigen immunoadsorbtion with a kit from the National Cancer Institute. Vector production and gene delivery were done in a biosafety level 2 environment. Vector-producing cells and transduced cells were fixed by a 30-min incubation in phosphate-buffered saline containing 4% paraformaldehyde before fluorescence-activated cell sorter (FACS) analysis on a Becton Dickinson FACScan.

In vitro transduction. In vitro transduction experiments were done in six-well plates (Costar). Filtered vector-containing medium was added 24 h after the cells (2×10^5 cells/well) had been seeded and was left until cells were analyzed 48 to 60 h later. Typically, the following amounts of p24 were used: 0.1 ng for titration of *lacZ* vectors by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining, 1 to 5 ng for luciferase assay, and 10 to 20 ng for β-galactosidase (β-Gal) enzyme assay and for titration of GFP vectors by FACS analysis. Multiplicities of infection can be estimated assuming that 1 ng of p24 corresponds to 1,000 to 5,000 transducing units (TU).

HIV-1 infection. Vesicular stomatitis virus (VSV) G-pseudotyped HIV-1 particles were generated by transfection into 293T cells of the plasmid HXBH10ΔenvCAT, a Vpu-positive HIV-1 derivative with a deletion in *env* and a chloramphenicol acetyltransferase (CAT) gene in place of *nef* (a kind gift of H. Göttlinger, Dana-Farber Cancer Institute), and pMD.G. The conditioned medium was collected and filtered, and 50 ng of p24 antigen was used to infect overnight 10^6 SupT1 cells. Infected cells were assayed by p24 immunostaining or CAT assay to demonstrate similar extents of infection (not shown).

In vivo gene delivery. Vector particles were concentrated from filtered supernatants by two rounds of centrifugation. Fisher 344 male rats weighing approximately 220 g were obtained from Harlan Sprague-Dawley and housed in accordance with published National Institutes of Health guidelines. All surgical procedures were performed with the rats under isofluorane gas anesthesia with aseptic instruments. Two microliters of lentivirus vector in phosphate-buffered saline was injected slowly (0.5 μl per min) into the striatum under stereotaxic guidance. One month after the injection, the animals were sacrificed and the brains were analyzed for GFP expression by immunocytochemistry. The primary anti-GFP antibody was purchased from Clontech and used at a 1:1,000 dilution. Biotinylated rabbit anti-goat secondary antibody, streptavidin conjugated to horseradish peroxidase, and the VIP chromagen kit were from Vector.

RESULTS

A 400-nucleotide-long deletion in the U3 region of the HIV-1 3' LTR does not affect vector titers. The upstream sequence element essential for polyadenylation of the HIV-1 genomic RNA has been mapped to a region situated between the TATA box and the beginning of the R region (26, 27). In contrast, all of the major determinants responsible for regulating the HIV-1 LTR promoter activity (including the so-called negative response element, the two NFκB and the NF-ATc binding sites, the three SP1 binding sites, and the TATA box) are located within the boundaries of a 400-nucleotide-long *Eco*RV-*Pvu*II fragment which does not overlap with the upstream sequence element (Fig. 1). Based on this premise, this fragment was deleted from the 3' LTR of the pHR' CMVlacZ plasmid used to generate a β-Gal-expressing HIV-based vector. In the resulting pHR'CMVlacZSIN-18 construct, only 53 nucleotides were left in U3: 35 nucleotides upstream of the *Eco*RV site to preserve efficient recognition and processing by integrase and 18 nucleotides downstream of the *Pvu*II site to govern polyadenylation. Transducing particles were produced by transient transfection of three plasmids into 293T cells as previously described (31): the multiply deleted packaging construct pCMVΔR8.91, which encodes Gag, Pol, Tat, and Rev; a plasmid expressing the surface glycoprotein (G) of VSV; and the vector DNA itself, in this case either the original pHR'CMVlacZ plasmid or its U3 deletion pHR' CMVlacZSIN-18 version. The two vectors gave comparable titers as measured with 293T cells as targets: 1,476 ± 232 TU per ng of p24 capsid antigen for the SIN-18 vector and 1,544 ± 126 TU/ng of p24 for the control. The blue color following X-Gal treatment appeared already after 3 h in cells transduced with the SIN-18 vector, whereas cells transduced with full-length U3 vector scored positive only after 6 to 8 h. This suggested that LacZ expression was higher when the flanking

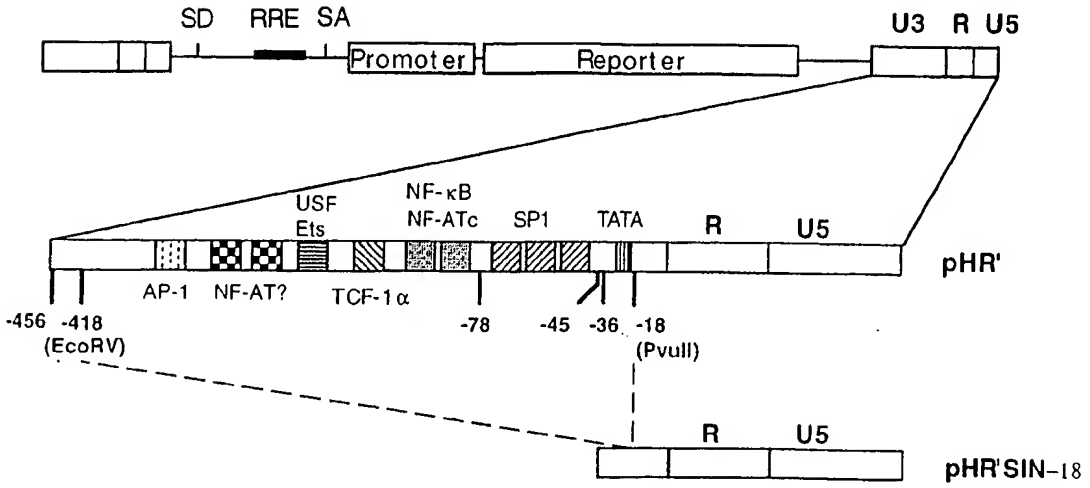

FIG. 1. Structure of SIN HIV-derived vectors. A schematic representation of an HIV-1 vector with enlarged 3′ LTR to show the binding sites for differents transcription factors on U3 is shown (not to scale). Although the 3′ LTR is depicted, the nucleotide numbering refers to the cap site at the beginning of R as +1 as for a 5′ LTR. Position −418 is the 5′ limit of all deletions; positions −78, −45, −36, and −18 indicate the 3′ limits of the different deletions described in the text. The deletion generating the SIN-18 vector created a novel *Bgl*II site. Details on the nuclear factors binding U3 can be found in references 10, 15, and 22 and references therein. SD, splice donor; RRE, Rev-response element; SA, splice acceptor. The GenBank accession number for the wild-type 3′ LTR is M1991.

LTRs were deleted. The β-Gal activity in cells transduced by the SIN-18 vector was indeed twice that found in cells containing the parental vector, with the number of transduced cells being equal (Fig. 2). A similar observation was made with a pair of full-length and SIN-18 luciferase-expressing vectors, although in this case the number of transduced cells could not be determined (not illustrated).

Vectors with less extensive U3 deletions were also generated (Fig. 1). In the SIN-36 and SIN-45 vectors the TATA box is intact, while in the SIN-78 vector the TATA box and the three SP1 binding sites are preserved. All U3 deletion vectors had transducing abilities that were comparable to that of their full-length U3 parent in both HeLa cells and peripheral blood lymphocytes (Table 1).

Unlike plasmids previously engineered to produce SNV-based SIN vectors (6), pHR′SIN plasmids have no polyadenylation signal downstream of the U3 deletion LTR to remedy a possible weakness of the RNA 3′ end processing. Thus, similar titers for SIN and regular vectors suggested that even the LTR with the most extensive U3 deletion had retained good polyadenylating activity (Table 1). Polyadenylation of the SIN-derived transcripts was also assumed to be efficient in target cells because of the good expression of the transgene in the SIN setting.

Transcriptional impact of U3 deletions. As a first approach to determine the effects of the various U3 deletions on vector-derived RNA production in target cells, HeLa cells transduced with regular or SIN GFP-expressing vectors were subjected to Northern blot analyses, using a GFP probe capable of detecting transcripts produced from both the LTR and the phosphoglycerate kinase (PGK) internal promoter (Fig. 3). A small amount of spliced, LTR-derived RNA was detectable in HeLa cells transduced with the full-length U3 vector, despite the absence of Tat (lane 1). This residual LTR promoter activity

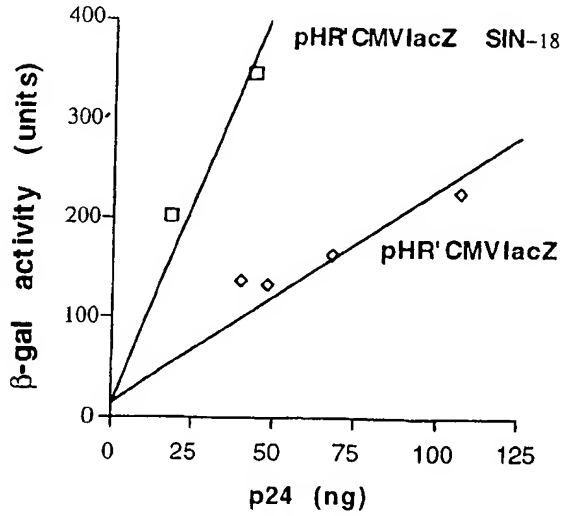

FIG. 2. Expression of a *lacZ* transgene delivered by SIN or full-length LTR vectors. 293T cells were transduced with equal volumes (200 μl) of two HR′CMVlacZSIN-18 or four HR′CMV *lacZ* vector stocks. Titers (TU per nanogram of p24) were similar for all stocks. β-Gal activity (in arbitrary units) at 48 h postinfection is plotted against the amount of p24 in vector stocks. Cells transduced with SIN-18 vectors express more than twice as much β-Gal per nanogram of p24 than cells transduced with full-length LTR vectors.

TABLE 1. Deletions in the U3 region of the HIV-1 LTR do not affect vector titers[a]

Vector	Titer on HeLa cells[b] (10^6 TU/ml)	Transduction of human PBLs[c] (%)
RRLPGK-GFP	18 ± 2.4	50.75 ± 1.12
RRLPGK-GFPSIN-18	14.5 ± 2.7	59.27 ± 0.90
RRLPGK-GFPSIN-36	13 ± 2.1	55.86 ± 2.26
RRLPGK-GFPSIN-45	12.7 ± 1.1	54.38 ± 1.16
RRLPGK-GFPSIN-78	12.8 ± 1.2	55.59 ± 1.20

[a] Fluorescent cells were scored by FACS analysis 6 days after transduction. Results are averages and standard deviations for duplicate determinations for an experiment representative of three performed.

[b] The end point titer was determined by multiplying the number of fluorescent cells by the vector dilution. Samples were selected from the linear portion of the vector dose-response curve.

[c] Percentage of fluorescent human peripheral blood lymphocytes (PBLs) after infection of 10^6 cells with 1 ml of vector-containing medium.

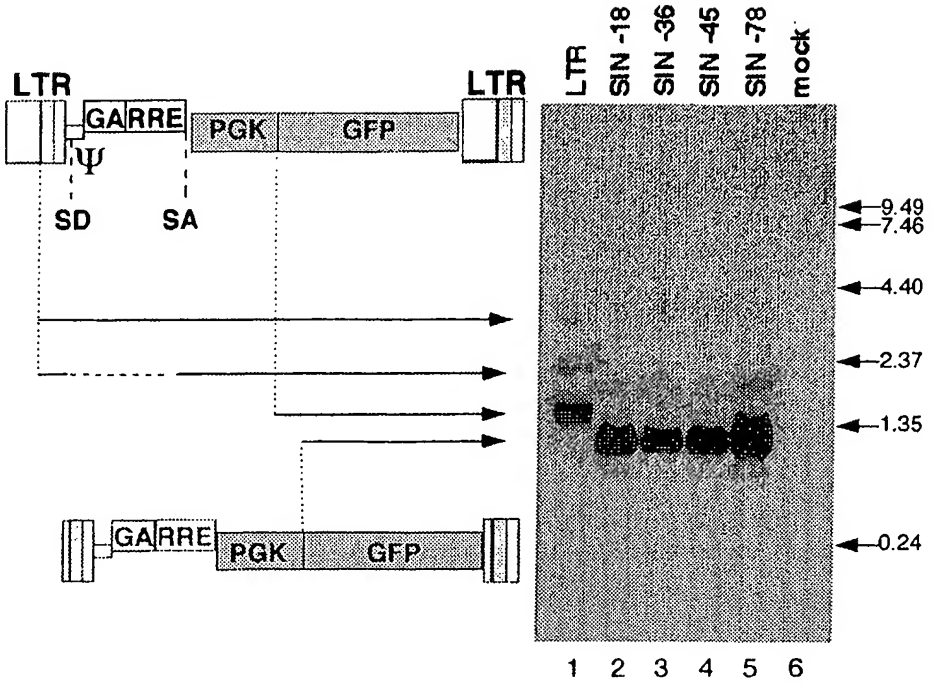

FIG. 3. Northern blot analysis of vector-derived transcripts in transduced HeLa cells. Total RNA was extracted from HeLa cells transduced with an RRLPGK-GFP vector (lane 1) or with its SIN versions SIN-18 (lane 2), SIN-36 (lane 3), SIN-45 (lane 4), and SIN-78 (lane 5). In lane 1, three bands with the sizes expected for the LTR-derived transcripts (unspliced and spliced) and the PGK-derived transcripts are visible. As expected for HeLa cells, transcription was initiated much more frequently at the internal PGK promoter than at the 5′ HIV-1 LTR. In lanes 2 to 5, transcripts derived from SIN vectors are 340 to 400 nucleotides smaller than the corresponding transcripts in lane 1. RNA initiated at the HIV-1 LTR is detectable in lane 5 but not in lanes 2 to 4. Positions of molecular size markers (in kilobases) are indicated on the right. Ψ, encapsidation signal; SD, splice donor; SA, splice acceptor.

was also observed with a deletion limited to sequences upstream of the SP1 sites, in the SIN-78 vector (lane 5). However, no LTR-driven transcript was detected with any of the SIN vectors lacking the SP1 binding sites (lanes 2 to 4).

The high sensitivity of the luciferase activity assay and the strong stimulation of HIV-1 LTR promoter activity by Tat were exploited to analyze more accurately the transcriptional activities of the SIN vectors. For this, vectors containing the luciferase cDNA without an internal promoter, that is, those in which transgene expression is controlled exclusively by the HIV-1 5′ LTR, were used. Luciferase activity was measured in HeLa-tat, HeLa, 293T, and NIH 3T3 cells infected with normalized amounts of HR′luciferase or HR′luciferaseSIN-18 vectors (Table 2). With the full-length U3 vector, a strong production of luciferase was detected in HeLa-tat cells, while it was moderate in 293T cells and weak in HeLa and NIH 3T3 cells. The results obtained with 293T cells may reflect the presence in these cells of the adenovirus early protein E1A (24), which is known to stimulate HIV-1 LTR promoter activity (16). The U3 deletion present in HR′luciferaseSIN-18 resulted in a 350-fold reduction of luciferase activity in HeLa-tat and 293T cells. The very low levels of luciferase in HeLa-tat cells transduced with HR′luciferaseSIN-18 confirmed the transfer of the U3 deletion to the 5′ LTR and the minimal transcriptional activity of the U3 deletion LTR even in the presence of Tat. However, the SIN-18 vector still induced higher levels of luciferase in HeLa-tat cells than in HeLa cells. As the deletion abrogates transcription from the upstream LTR, this raised the possibility that the U3 deletion was repaired at a low but detectable frequency (see below) or that a promoter trap mechanism was enhanced by the presence of Tat in target cells. To investigate this point further, vectors expressing β-Gal without an internal promoter were used. In HeLa-tat cells, after normalization for p24 content of the in-

TABLE 2. Promoter activity of the HIV-1 LTR with a U3 deletion

Vector	Luciferase activity (RLU) in transduced cells[a]			
	HeLa-tat	HeLa	293T	NIH 3T3
HR′luciferaseSIN-18	245 ± 66	96 ± 12	34 ± 13	4.5 ± 0.6
HR′luciferase	87,553 ± 9,038	1,888 ± 272	11,613 ± 1,733	349 ± 71
ΔU3/wild-type U3[b]	0.0028	0.05	0.0029	0.012

[a] Two HR′luciferaseSIN-18 and two HR′luciferase vector stocks were independently produced and used to transduce in parallel and in duplicate HeLa-tat, HeLa, 293T, or NIH 3T3 cells. Under the conditions used (2 ng of p24 with 2×10^5 cells), the multiplicity of infection can be estimated to be about 0.01. For each vector type the results for the two stocks were averaged. The given relative light unit (RLU) values are for 20 μl of a 200-μl protein extract. The luciferase activity in each protein extract was assayed in duplicate and averaged, and the background value for each cell type was subtracted. Results are means and standard deviations.

[b] Ratio of the activities obtained with the two types of vectors.

TABLE 3. Relative expression of the luciferase gene delivered by U3 deletion and full-length LTR HIV-1 vectors

Target cells	Relative expression[a] using the following internal promoter:		
	CMV[b]		PGK[c] (dividing cells)
	Dividing cells	Gamma-irradiated cells[d]	
293T	2.39	2.10	2.2
HeLa	0.47	0.82	1.1[e]
F208	0.47	ND[f]	0.56
3T3	0.69	0.49	1.09[e]

[a] Luciferase activity (mean from two assays) was plotted against p24, and a linear regression was calculated for each type of vector by using Criketgraph as for Fig. 2. Values are the ratios of the slopes.

[b] Stocks of HR'CMVluciferase and HR'CMVluciferaseSIN-18 vectors were prepared in quadruplicate and used to transduce in parallel and in duplicate all four cell types.

[c] Stocks of HR'PGKluciferase and HR'PGKluciferaseSIN-18 were prepared in triplicate and used to transduce in parallel and in duplicate all four cell types.

[d] Eight thousand rads was delivered by a 3-min exposure to a ^{60}Co source.

[e] Transductions by U3 deletion and full-length LTR vectors were not significantly different (by Student's t test).

[f] ND, not determined.

ocula, the β-Gal-expressing HR'lacZ SIN-18 vector induced titers of 320 ± 11 TU/ml, compared with $4.1 \times 10^5 \pm 0.6 \times 10^5$ TU/ml for the control HR'LacZ vector. No positive cells were detected among HeLa cells exposed to 1 ml of either type of vector. The complete Tat dependence of β-Gal expression suggested that it resulted from U3 repair, although one could not completely exclude that Tat-mediated transcriptional activation enhanced promoter trapping. If transgene expression from the promoterless SIN-18 vector resulted entirely from U3 repair, then the frequency of this event, based on a comparison of the relative titers of the SIN-18 and wild-type vectors in HeLa-tat cells, was close to 1/1,000. It is important to recall, however, that in this case vector particles were generated by cotransfecting plasmids carrying a simian virus 40 (SV40) origin of replication in cells containing the SV40 large T antigen, a setting highly favorable for DNA recombination.

The potential impact of interactions between the LTR and the internal promoter was probed by evaluating the effect of deleting U3 on the production of a luciferase reporter expressed from two different internal promoters (cytomegalovirus [CMV] and PGK), using various cell types as targets (Table 3). Because the GC-rich sequence of the mouse PGK promoter contains only three ATG triplets, LTR-derived RNAs can be translated and can contribute to the expression of transgenes delivered by HR'PGK vectors. In contrast, the AT-rich CMV promoter sequence contains 17 ATG triplets, which impair the translation of LTR-derived RNAs. Despite this difference, with both promoters the SIN-18/wild-type U3 ratio was 2 in 293T cells, suggesting that in these targets the presence of a full-length LTR interferes with transcription from the internal promoter. The adenovirus early gene E1A, which is expressed in 293T cells, appeared to be responsible for this phenomenon, because a similar SIN-18/wild-type U3 ratio was noted in 293 cells, excluding a role for the SV40 large T antigen, and in a rat thyroid cell line immortalized with E1A but not in one immortalized with v-Raf (data not shown). However, the level of LTR activity per se did not seem to be the key factor in inducing promoter interference, because HeLa and HeLa-tat cells transduced with HR'CMV-GFP vectors expressed the same level of GFP even though the HIV-1 LTR is 50 times more active in HeLa-tat cells than in HeLa cells. With a PGK internal promoter, a moderate but consistently positive effect of the U3 deletion on transgene expression was observed in 293T, HeLa, SupT1, and 3T3 cells (Table 3 and data not shown).

Pattern of activation of SIN vectors following HIV infection of transduced cells. To investigate further the degree of transcriptional inactivation resulting from the various U3 deletions, human T lymphoid SupT1 cells stably transduced with wild-type or SIN PGK-GFP vectors were infected with envelope-defective, VSV G-pseudotyped HIV-1. Because the PGK promoter allows for the translational readthrough of LTR-derived transcripts, increases in GFP levels were used as a reflection of Tat-induced LTR activation. In cells containing the full-length U3 or the SIN-78 vector, GFP expression was stimulated following infection, while no such phenomenon was observed in cells transduced with the SIN-45, SIN-36, or SIN-18 vector (Fig. 4). Confirming the results obtained with HeLa-tat cells, these data indicate that also in an established T-cell line the HIV-1 LTR remains active despite the absence of all of the transcriptional elements located upstream of the SP1 binding sites and that the SIN-18 design abrogates this activity. Correspondingly, while full-length U3 and SIN-78 vectors could be rescued by HIV infection of transduced cells, recombine with the viral genome, and possibly be mobilized to new targets, these risks are theoretically alleviated by the use of the SIN-18 vector.

Efficient in vivo gene delivery by SIN vectors. The results described above indicated that the inactivation design did not interfere with the transduction of cellular targets in vitro by HIV-derived vectors. The performance of SIN vectors was assayed in an in vivo delivery model that demands high efficiency of gene transfer and expression. Vectors carrying a PGK-GFP expression cassette with full-length U3 or U3 with the sequence from position −418 to −18 deleted were concentrated to high titers, matched for particle content by p24 antigen, and injected bilaterally in the neostriata of two groups of three adult rats. The animals were sacrificed after 1 month, and serial sections of the brain were analyzed for GFP expression by fluorescence (not illustrated) and immunostaining (Fig. 5). Both types of vector transduced neurons very efficiently: GFP-positive cells were detected at a very high density throughout most of the striata of all the injected animals. The level of transgene expression directed by the SIN-18 vector appeared to be even higher than that obtained with the wild-type vector. These results provide evidence that a SIN HIV-derived vector is an efficient vehicle for in vivo gene delivery.

DISCUSSION

This study demonstrates that a large U3 deletion in the LTR of an HIV-1-based vector confers efficient self-inactivation without lowering the vector titer or impairing the expression of the transgene both in vitro and in vivo.

The HIV-1-based SIN vector presented here offers all of the previously claimed advantages of SIN retroviral vectors. First, the extensive U3 deletion of SIN-18 abolishes the viral promoter activity, thereby preventing the synthesis of full-length vector RNA in target cells. This results in minimizing the risk that a replication-competent retrovirus will emerge or that a cellular gene located immediately downstream of the 3′ LTR will be aberrantly expressed. Furthermore, the elimination of the LTR enhancer sequences in the SIN-18 design precludes the activation of a promoter located at a distance from the vector integration site. The so-called enhancer-less MLV vectors still have an active albeit attenuated viral promoter, and LTR-derived RNAs have been readily detected in transduced cells (11, 23). With such vectors, the spread of potential RCRs would not be limited to a single round of infection. Only the

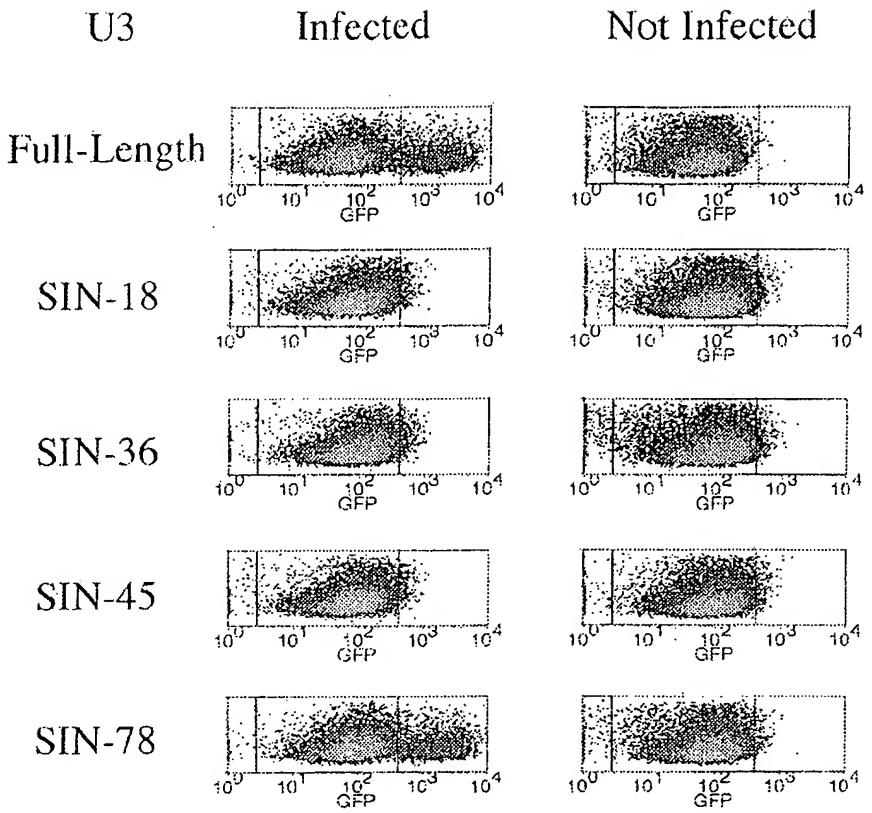

FIG. 4. Activation pattern of HIV-1 vectors following infection of transduced SupT1 cells by HIV-1. Human lymphocytic SupT1 cells were transduced at a high multiplicity of infection by HIV-derived vectors carrying a PGK-GFP expression cassette and either a full-length LTR or the indicated U3 deletion construct. Six days later, the stably transduced cells were infected with VSV G-pseudotyped HIV-1 or were mock treated, and 48 h later they were analyzed by FACS for GFP fluorescence. Infection with HIV-1 strongly enhanced the expression of GFP in cells transduced by a vector with a full-length U3 LTR or the −78 deletion construct, while it had no effect on cells transduced with vectors having larger U3 deletions. The left and middle quadrants represent the fluorescence of cells not transduced and transduced by the GFP vector, respectively. The right quadrant includes cells with increased GFP expression upon infection by HIV-1. The increased expression of GFP indicates activation of vector transcription from the LTR and is due to translational readthrough of the PGK promoter sequence upstream of the GFP cDNA (see text). The increased in fluorescence intensity was 30-fold for cells transduced by the full-length LTR and 21-fold for those transduced by the SIN-78 vector. The HIV-1 had a deletion in the envelope gene and was thus limited to one round of infection. Similar patterns of Tat responsiveness were observed when HeLa-tat cells were transduced with the various vectors (not shown).

SNV-based vector developed by Olson et al. (20) and a chimeric MLV-based vector developed by Hawley et al. (12) are transcriptionally disabled to an extent comparable to that obtained with the HIV vector described here.

The SIN design also prevents potential interferences between the viral LTRs and the internal promoter, a phenomenon which can have profound implications in gene therapy. For instance, it was observed that the liver-specific albumin promoter loses its tissue specificity when flanked by MLV sequences (28). The mechanisms of promoter interference remain poorly understood. According to the classical view of promoter occlusion (4, 21), the presence of an active upstream viral LTR should decrease the activity of the internal promoter. Results presented by Yee et al. (29) and Chen et al. (3) initially gave credence to the promoter occlusion theory, but this conception was subsequently challenged by two well-documented studies. Taking advantage of the fact that the MLV LTR is transcriptionally competent in fibroblasts but not in embryonic stem cells, Soriano et al. have shown that the activity of different internal promoters is influenced by the sequence but not by the levels of transcriptional activity of the upstream LTR (23). Another study with an MLV vector in which the U3 region was replaced by a tetracycline-inducible promoter showed that activation of the chimeric LTR did not affect transcription from an internal promoter (13). In our system, the activation of the upstream HIV-1 LTR by Tat also failed to induce the occlusion of a downstream CMV promoter. Nevertheless, the comparison of various SIN and full-length HIV vectors revealed some promoter- and cell-specific differences in the degree of promoter interference, but in all cases the magnitude of these effects was minimal. While LTR-induced transcriptional inactivation of transgenes in vivo has not yet been described for HIV-based vectors, it may be relevant for the transduction of novel targets. The SIN design might help to avoid such an occurrence. Furthermore, the creation of tissue-specific and inducible vectors will be significantly facilitated by the availability of the SIN vector described here, which allows both the delivery of an internal expression cassette without flanking sequences that might influence its transcription and the swapping of novel enhancer-promoter sequences in the place of the deletion.

It is possible that the SIN HIV-1 vector described here underwent a repair of the U3 deletion at a maximal frequency of 1/1,000. This is much lower than that reported for the first generation of SNV- and avian leukosis virus-based U3 deletion vectors (9, 19). Moreover, it is likely that in our system the bulk

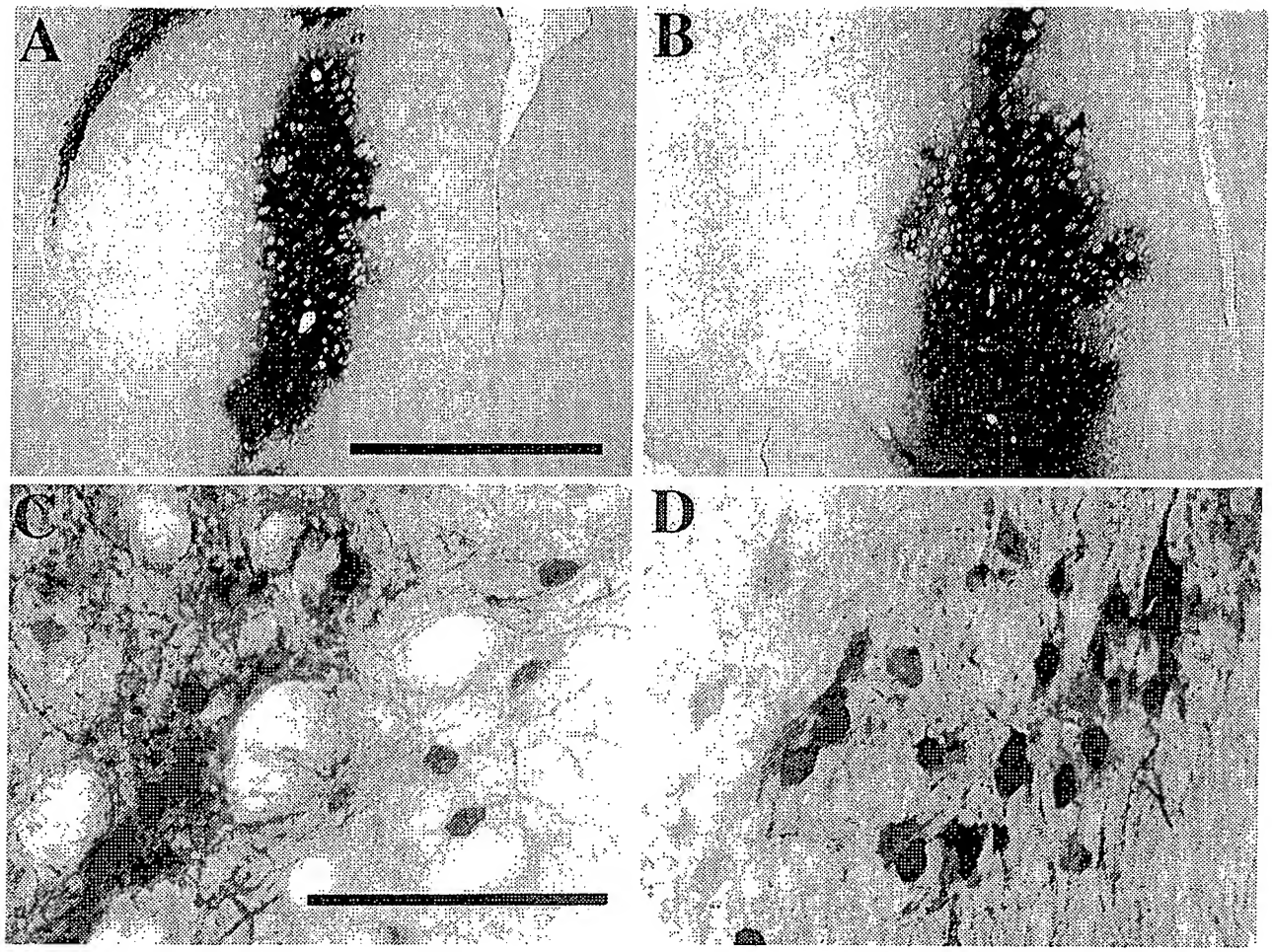

FIG. 5. In vivo transduction of GFP into neurons by SIN or full-length LTR vectors. HIV-1 vectors carrying a PGK-GFP expression cassette with the full-length U3 region (A and C) or the −18 deletion construct (B and D) were concentrated by ultracentrifugation and normalized for particle content prior to injection into the corpora striata of adult rats. One month after injection, brain sections were stained for immunoreactivity to the GFP protein. Both types of vectors transduced neurons very efficiently. The SIN vector often appeared to achieve a higher level of transgene expression. A representative section close to the injection site is shown for one of six injected striata per vector. Bars in panels A and C, 2 and 0.1 mm, respectively (magnifications are the same for panels B and D, respectively).

of the repairing events occurred by recombination of the transfected plasmids. Documenting exactly what this frequency is in the current system is of little relevance, because only vectors produced from stable packaging cell lines will ultimately be considered for clinical use. When such cell lines become available for HIV vectors, it is likely that their SIN versions will exhibit the same low repair frequency as the newest generation of SNV-based SIN vectors (20).

The SIN design slightly increases the packaging capacity of HIV-based vectors by removing 400 bases of virus-derived sequence. Experiments performed with HIV-1 derivatives harboring the cDNAs of selectable markers in place of *nef* have demonstrated that viruses with a genome of more than 11 kb of RNA can maintain a full infectivity (25). In its current configuration, the SIN-18 vector contains approximately 1.7 kb of HIV-specific *cis*-acting sequence. Assuming that an internal promoter will occupy on average 500 bases, HIV-based vectors should be able to accommodate transgenes of at least 8.8 kb.

Finally, from a biosafety point of view, the newest generation of HIV-1-based vectors appears to be particularly reliable. Major improvements were brought to the original packaging system, first by deleting *vif*, *vpr*, *vpu*, *env*, and *nef* (31) and subsequently by removing *tat* and by expressing the *gag-pol* and *rev* genes from split genomes (7). Of note is that the strict Rev dependence of HIV-1 allows a distribution of the constituents of the vector-packaging system into more independent entities than is possible with MLV-based vectors. Here, we further demonstrate that a SIN HIV-based vector retains all of the properties of its full-length parent. When produced by packaging cell lines incorporating all of these safeguards, HIV-1-based vectors should meet the most stringent safety requirements for clinical applications.

ACKNOWLEDGMENTS

We thank H. Göttlinger for the CAT-expressing HIV-1 derivative and W. Haseltine and E. Terwilliger for HeLa-tat-III cells, obtained through the NIH AIDS Research and Reagent Reference Program.

This work was supported by a grant from the Swiss National Science Foundation and by a professorship from the Giorgi-Cavaglieri Foundation to D.T. R.Z. was the recipient of a fellowship from the Swiss National Science Foundation.

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Exhibit 2

Deisseroth, *Clinical Cancer Research, 5*:1607-1609 (1999); Office Action dated June 18, 2004, page 7.

Editorial

Clinical Trials Involving Multidrug Resistance Transcription Units in Retroviral Vectors

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Cowan *et al.* (1) have reported in this issue of *Clinical Cancer Research* the results of a clinical trial that was designed to evaluate the feasibility of using retroviral vectors to transfer the *MDR-1*[2] chemotherapy resistance genes to normal hematopoietic cells to protect them from the effects of chemotherapy.

The *MDR-1* gene codes for p-glycoprotein, an ATP-dependent membrane protein known to transport many of the complex alkaloid chemotherapy agents, such as the anthracyclins, the periwinkle alkaloids, and the epidophyllotoxins from inside to the outside of cells. By reducing the achievable intracellular concentrations of each of these drugs, the p-glycoprotein protects the cells from the toxicity of these drugs. The presence of high levels of the p-glycoprotein is associated with high levels of resistance to many epithelial tumors. In addition, Chaudhary and Roninson (2) had shown that the level of expression of the *MDR-1* gene decreases as hematopoietic cells matured in the marrow. This data provided one possible mechanism through which tumors can acquire resistance to multiple chemotherapy agents through the overexpression of a single gene. In addition, it provided one of many possible mechanisms through which immature hematopoietic cells can survive doses of chemotherapy that are lethal to hematopoietic cells belonging to more mature stages of hematopoietic differentiation. Finally, this data suggested to many that the transfer of the gene for the *MDR-1*, in a transcription unit controlled by a promoter that exhibited high levels of expression in mature as well as immature hematopoietic cells, might decrease the toxicity of chemotherapy by conferring resistance to doses of chemotherapy otherwise associated with life-threatening levels of chemotherapy.

Ueda *et al.* (3) were the first to test this concept by constructing transgenic mice in which the marrow cells were expressing high levels of the MDR-1 cDNA. These mice showed no hematopoietic toxicity to doses of chemotherapy that otherwise would cause life-threatening hematopoietic toxicity. In addition, there was no observable change in the maturation of the hematopoietic cells over a period of several years, in the presence of high levels of p-glycoprotein.

Following the lead of Ueda *et al.* (3), several groups then presented the results of using Maloney Leukemia Virus- and Harvey Sarcoma virus-based retroviral vectors to transfer the human *MDR-1* gene into the hematopoietic cells of mouse marrow isolated from 5-fluorouracil-treated donors (enriched in immature cells). The transduction conditions involved either cocultivation of the producer cells lines with the hematopoietic cells or exposure of the hematopoietic cells in suspension or on stromal monolayers to cell-free supernatants of the viral particles. After transplantation of these vector modified cells into lethally irradiated recipient mice, it was shown that: (*a*) the transplanted MDR-1-transduced hematopoietic cells were resistant to much higher concentrations of the drug in clonogenic progenitor assays than in the cells of unmodified donors (4–7); (*b*) the administration of posttransplant chemotherapy was associated with increased levels of the vector-modified cells in the animals transplanted with MDR-1 vector-transduced cells (4); and (*c*) the MDR-1 vector transduced cells were capable of serially transplanting up to six successive cohorts of mice, whereas unmodified cells would only successfully serially engraft three successive cohorts of irradiated mice (6–7). The hematopoietic maturation appeared normal in all of these transplant experiments, and no evidence of myelodysplastic states were associated with this vector modification. A cryptic splice acceptor side was also discovered in the *MDR-1* gene, which resulted in a variable percentage of the transductants containing functionless truncation mutants of p-glycoprotein (4, 6–7). The most encouraging aspects of these preclinical trials were the long-term engraftment and the increases in the levels of vector-modified cells during the administration of posttransplantation chemotherapy, which compensated, in part, for the low percentage of transplantable hematopoietic cells that were MDR-1 vector positive immediately after transplant (4).

These data led to an interest all over the world in testing whether such vector delivery systems could be used to deliver MDR-1 transcription units to patients with epithelial malignancies. The interest in developing such programs was further heightened by the advent of the taxanes, which were associated with dose-limiting hematopoietic toxicity, along with neurotoxicity.

In the initial trials, institutions in the United States [NIH (Bethesda, MD), Columbia University (New York, NY), and the University of Texas M.D. Anderson Cancer Center (Houston, TX)] secured approval from the NIH Recombinant DNA Advisory Committee and the Federal Drug Administration to carry this concept into the clinic (1, 8–10). The conditions of transduction in each case involved incubation for several days in medium supplemented with serum and late-acting hematopoietic growth factors. All three groups used the autologous transplantation model for testing of the engraftment of MDR-1 vector-modified cells (1, 7–9), and two of the trials studied, in addition, the effect of posttransplant chemotherapy in the context of the transplantation of MDR-1 vector-modified cells (1, 8, 10).

The first trial to be published, which involved 20 patients, showed only short-term engraftment of vector-modified cells

Received 3/30/99; revised 4/3/99; accepted 4/6/99.

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[2] The abbreviation used is: MDR, multidrug resistance.

and that *in vitro* transduction of the clonogenic progenitor cells was not predictive of the presence of posttransplant MDR-1 vector-positive hematopoietic cells (7). This data suggested that at least the subset of the clonogenic progenitors, which were transduced, did not have significant engrafting capability (8, 10). This result (8) confirmed earlier mouse studies by Uchida *et al.* (11), which had suggested that the clonogenic progenitors had limited self renewal capability.

The next group to publish the results of their MDR-1 vector modification trial also showed only very short-term engraftment of the MDR-1 vector-mediated cells after transplant (9). The next publication showed that the delivery of posttransplant chemotherapy immediately after engraftment, in the context of the MDR-1 chemoprotection trial, converted some breast cancer patients from a partial response after transplant to a complete clinical response (10). Two of the patients achieving partial response after the pretransplant intensive chemotherapy and autograft, and then a complete response from the posttransplant chemotherapy, are still disease-free in unmaintained remissions over 3 years after the initial transplant (10). Thus, although the vectors used for the MDR-1 delivery were not as successful as anticipated, the results of this trial suggested that genetic chemoprotection could be of potential utility in the use of high-dose therapy and autografts.

Cowan *et al.* (1) now report the results of their trial, which involved seven patients transplanted with MDR-1 vector-modified cells. Their results show that only short-term engraftment with MDR-1 vector-modified cells was seen after transplant. However, one of their patients exhibited stable, but low-level, engraftment with the MDR-1 vector-positive cells for several months after transplant (1). A normal pattern of hematopoietic differentiation was maintained in this patient. Cowen *et al.* (1) discussed the reasons for the short-term, but not long-term, engraftment of MDR-1 vector-positive cells in the marrow of patients participating in the human MDR-1 vector transplant trials. After the initiation of these clinical trials, multiple groups have shown that the conditions used in the early three MDR-1 chemoprotection trials (interleukin-3, stem cell factor, and serum) led to induction of maturation of the early hematopoietic stem cells and decreased levels of engraftment of the *in vitro*-manipulated cells (12). Orlic *et al.* (13) have shown that the receptors needed by the vectors to infect the target cells are not represented on early human stem cells to the degree that they are present on mouse stem cells, or more mature human hematopoietic cells. The transcriptional regulatory elements of the Maloney and Harvey vectors undergo methylation in the early hematopoietic cells, which could have suppressed the level of expression of the *MDR-1* genes in the stem cells. The doses of posttransplant chemotherapy used to attempt to create a selective advantage for the MDR-1 vector-modified cells were set for safety reasons at too low a level to confer a selective advantage on cells that were positive for the *MDR-1* vector transgene.

Several developments have occurred in vector design and in systems available for the *in vitro* incubation of human hematopoietic cells, which have potentially addressed many of the problems that may have contributed to the failure to achieve long-term engraftment and chemoprotection in these early trials:

(*a*) *Ex vivo* serum-free culture conditions that support the survival and self renewal of stem cells have been developed (14, 15), which will enable future trials to avoid the conditions used for transduction in the early MDR-1 trials (incubation in serum containing medium supplemented with late-acting growth factors, such as interleukin-3, in the presence of SCF), which have been shown to induce maturation of stem cells and to, therefore, reduce their self renewal potential in transplantation settings;

(*b*) The development of pseudotyping of the retroviral vectors have allowed newer vectors to be created, which display ligands on their surface for which receptors are present on stem cells. These vectors show much higher transduction frequencies of the stem cells than obtainable in earlier studies;

(*c*) Baum *et al.* (16) have developed vectors in which the introduction of transcriptional regulatory elements of the mouse embryonic stem cell virus, which is not methylated in stem cells, contribute to much higher levels of expression of the vector transgenes in hematopoietic stem cells than was the case with the Maloney and Harvey vectors used in the early MDR-1 trials in which the transcriptional elements of the virus are inactivated by methylation and, therefore, the expression of the retroviral transgenes are only of short duration;

(*d*) The lentiviral vectors (17) have been developed, which retain the ability to transport the vector cDNA through the intact nuclear membrane of the nondividing cell, whereas the Maloney and Harvey vectors could not deliver their cDNA through the intact nuclear membrane of the nondividing cell (this limited the transduction of reconstituting early hematopoietic stem cells, most of which are nondividing, in the early trials). These lentiviral vectors have been shown to result in modification of at least 80% of the CD34+CD38− cells, and the retroviral vectors developed in serum-free medium have been shown to result in the modification of 50% of these cells;

(*e*) An entire series of additional chemoprotection transcription units, in addition to MDR-1, have been introduced into vectors and shown to protect hematopoietic cells;

(*f*) Newer and more powerful growth factors, which are specific for the early cell, have been cloned (such as FLT 3 ligand), which are now available for creating serum-free *in vitro* transduction conditions that preserve the integrity of the hematopoietic stem cells;

(*g*) Cornetta and his coworkers (18) have used the fibronectin fragment of David Williams to increase the transduction frequency of hematopoietic cells and the percentage of MDR-1 vector-positive cells posttransplant;

(*h*) Several new vector and animal models are now available for the study of the engraftment capabilities of genetically modified stem cells: the NOD X SCID transplant model (17) and the fetal sheep model of Zanjani (19, 20). Experiments such as serial transplantation need to be carried out so that the true self renewal potential of these modified cells can be assessed.

The early MDR-1 vector modification studies (1, 8–10), such as that of Cowan *et al.*, published in this issue of *Clinical Cancer Research* (1), have clarified the logistics and the hurdles that need to be overcome for genetic modification of hematopoietic stem cells to become relevant to clinically important end points. Although old questions remain about the ability to ultimately succeed in stem cell gene therapy and new questions have arisen about the impact of overexpression of MDR-1 transcription units in hematopoietic cells (21), the developments

in the field of virology and the biology of the hematopoietic stem cell, which have occurred since the inception of these trials, may make this dream a reality in the very near future and lead to a new round of preclinical trials to test the feasibility of using vector-mediated gene transfer as a means of reducing the risk and cost of administering chemotherapy for the treatment of epithelial neoplasms, and for correcting constitutional and acquired abnormalities of the hematopoietic cells.

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Exhibit 3

Chang *et al., Gene Therapy 6*:715-728 (1999); Office Action dated June 18, 2004, page 8.

Gene Therapy (1999) 6, 715–728
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Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system

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Lentiviruses infect both dividing and nondividing cells. In this study we characterized a lentiviral vector system consisting of a packaging vector (pHP) and a transducing vector (pTV) derived from a recombinant human immunodeficiency virus type 1 (HIV-1). In pHP, the long terminal repeats (LTRs), the 5' untranslated leader and portions of the env and nef genes were deleted. The leader sequence of pHP was substituted with a modified Rous sarcoma virus (RSV) 59 bp leader containing a mutated RSV gag AUG and a functional 5' splice site. The pHP construct was found to direct Gag-Pol synthesis as efficiently as wild-type HIV-1. The pTV construct contains sequences required for RNA packaging, reverse transcription and integration, but lacks viral genes. Co-transfection of pHP, pTV and a vesicular stomatitis virus G (VSV-G) envelope plasmid produced vectors at titers of 10^5–10^6 transducing units per milliliter in 48 h. Replication-competent virus (RCV) was not detected when deletions were made in the env gene in pHP. The ability of this vector system to transduce dividing and nondividing cell in vitro and in vivo was also demonstrated. Compared with a Moloney murine leukemia virus (MLV) vector, the HR/TV vectors transduced human muscle, kidney, liver-derived cell lines and CD34+ primary hematopoietic progenitor cells more efficiently. Although the levels of the pTV transgene expression were high soon after transduction, the expression tended to decrease with time due either to the loss of proviral DNA or to the inactivation of promoter activity which was found to be cell type-dependent. Analyses of extrachromosomal DNA showed that the unintegrated proviral DNA of lentiviral vectors survived much longer than that of the retroviral vectors. We demonstrate that the HR/TV vector is capable of high efficiency transduction and that long-term expression of lentiviral vectors is dependent on target cell type, the internal promoter and the transgene itself in the transducing vector.

Keywords: *HIV; lentivirus; vector; MLV; VSV-G*

Introduction

Efficient, long-term gene transfer is essential for clinical applications of gene therapy. Viral vectors are capable of efficiently transducing genes into target cells because they can bind host cell receptors and exert viral mechanisms for efficient gene expression. Retroviral vectors derived from MLV are well established and have been a useful tool for gene delivery.[1,2] Other members of the retrovirus family, including HIV, human foamy virus (HFV) and feline immunodeficiency virus (FIV), have gained much attention recently in that infection of these viruses is not limited to actively dividing cells.[3–5] The restricted host cell tropism of lentiviruses such as HIV and FIV has been overcome by using alternative pseudotyping viral envelopes such as amphotropic MLV env or vesicular stomatitis virus G (VSV-G) glycoproteins.[6]

Extensive studies of HIV have laid significant groundwork for the development of HIV-based lentiviral vectors. Shimada *et al*[7] have described a recombinant HIV-1-based gene transfer system that efficiently targets human CD4+ cells. This system consists of a packaging construct that expresses HIV-1 gag-pol and env genes and a transducing construct that expresses a reporter neo^R gene. Using a three component vector system that included a third VSV-G envelope pseudotyping plasmid, Naldini *et al*[8] were able to expand the host cell types that could be targeted by HIV-1 vectors. Similar vector systems have recently been described that use HIV-2 and FIV constructs.[5,9]

The development of a useful lentiviral vector system must take into consideration basic lentiviral genetics and safety issues. For instance, while the amount of HIV sequence present in the vector is directly correlated with the risk of generating RCV, it is still not clear how much of the viral genome can be deleted during vector construction without adversely affecting the efficiency of the vector.[4] The first generation of lentiviral vectors should be constructed without any of the nonessential viral genes (eg accessory genes such as *vif, vpr, vpu, nef*) to reduce the risk of pathogenesis and without any sequences not required for vector functions (eg LTRs and *env* gene of the packaging vector constructs and gag-pol and env reading frames of the transducing vector constructs) to reduce the possibility of homologous recombination. Detailed mutational analyses of lentiviral genome will be necessary in order to determine what the minimal requirements are for an efficient and safe vector system.

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Received 28 July 1998; accepted 17 December 1998

To date, little information is available concerning the range of human cell types that can be efficiently and stably transduced by lentiviral vectors. In addition, the advantage of lentiviral vectors over conventional retroviral vectors has not been well characterized. To investigate these issues, we generated a recombinant HIV-1 vector system with multiple safety features based on a helper construct pHP and a transducing vector construct pTV. The possibility of producing replication competent virus (RCV) was carefully examined. The transduction efficiency of the HP/TV vector and a conventional MLV vector was studied using different human cell types including TE671 (muscle), 293T (kidney), HepG2 (liver), neuronal stem cells and primary CD34 hematopoietic progenitor cells and nonhuman primary rat neural and muscle cells. Transduction efficiency was assayed over short and long duration in tissue culture. The safety, expression kinetics, duration and integration status of this lentiviral HP/TV vector system are presented.

Results

Construction of recombinant HP/TV vectors

The two lentiviral constructs, pHP, which directs the synthesis of necessary viral proteins for virion assembly and pTV, which serves as a gene transducing vehicle for foreign gene delivery (see Figure 1), were derived from a LTR-modified recombinant HIV-1 plasmid pNL4-3 with multiple modifications for safety reasons.[10] pHP-1 contains a recombinant cytomegalovirus immediate-early (CMV-IE) enhancer/promoter HIV-TAR element which replaces the 5′ LTR of pNL4-3. The entire untranslated 5′ leader sequence, *nef*, and the 3′ LTR of pNL4-3 were also deleted in pHP-1 (Figure 1a). The HIV-1 5′ untranslated leader sequence was replaced with a modified Rous sarcoma virus (RSV) 59 nt leader sequence containing a major splice donor site, a mutated RSV gag AUG and a preferred eukaryotic translation initiation sequence (-CCACCATG-) for the HIV-1 Gag synthesis (Figure 1c). The *nef* gene in pNL4-3 was replaced by the bacterial *gpt* selective marker gene, and the 3′ LTR was replaced by the SV40 polyadenylation signal. To prevent RCV production, the *env* gene was deleted by Bal31 exonuclease digestion at the *Nhe*I site. Two variants of env-deleted pHP-1 were generated and analyzed, pHP-1dl.2 (with a 2-nt deletion) and pHP-1dl.28 (with a 28-nt deletion). An alternative pHP construct, pHP-VSVG, was generated by inserting the VSV-G envelope gene between the *env* AUG and the *Nhe*I site in gp120 and introducing mutations in *vpr*, *tat*, and *vpu* (Figure 1a).

The transducing constructs (pTV) were made from pNL4-3 by deleting sequence extending from the middle of *gag* to the middle of *env*. A reporter gene cassette containing a heterologous enhancer/promoter directing a reporter gene was inserted in the *nef* region of the pTV vector as illustrated in Figure 1b.

Lentiviral vector production, transduction and RCV assay

As safety is a major concern with HIV-derived vectors, the HP/TV vector system has been developed to minimize the possibility of homologous recombination and RCV production. To examine vector efficacy and RCV production, human rhabdomyosarcoma TE671 cells were co-transfected with the following three plasmids, a pHP construct, pHP-1 or pHP-VSVG, an envelope expression plasmid pHEF-VSVG and a pTV construct, pTVΔSVneo, pTVΔSVhyg, or pTVΔCMV-nlacZ (Figure 1). Since pHP-VSVG does not have a functional *tat* gene, a tat plasmid was also included when pHP-VSVG was tested. The resulting vector titer was determined by titration on TE671 cells using transfected culture supernatants. The reporter genes, *neo*, *hyg* and *lacZ*, were assayed by selecting resistant cell colonies using either G418, hygromycin B, or by colorimetric staining for β-galactosidase activity, respectively. For the detection of RCV, the transfected TE671 cells were co-cultured with MT4 cells, one of the most susceptible cell lines to HIV-1_{NL4-3} infection. After co-cultivation, MT4 cells were monitored for syncytium formation and culture supernatant was subjected to HIV-1 reverse transcriptase (RT) assay. To increase the sensitivity of detecting RCV, fresh MT4 cells were added to the transfected culture weekly and 1 month after co-culture, MT4 cells were fixed and immunostained using anti-HIV antibodies as described in Materials and methods. Results of this experiment are summarized in Table 1. HP/TV co-transfection successfully produced viral vectors from all three pTV reporter constructs suggesting that the transducing vectors could be packaged into virus particles. However, RCV was detected in all transfected cultures except for pHP-VSVG. To generate RCV from co-transfections of pHP-1 and pTVΔ, at least two homologous genetic crossovers must occur, one in *gag* and the other in *env*. These results demonstrate that recombinant RCVs could arise from co-transfection of these vectors and that the co-culture and immunoassay provide a sensitive means for detecting RCVs.

Two nucleotide deletion in env prevents RCV production

To improve the safety, the *env* gene was deleted in pHP and two deletion constructs were generated, pHP-1dl.2 and pHP-1dl.28. TE671 co-transfection was performed as described above. The wt HIV-1 plasmid pNL4-3 was included for comparison. RCV assay was performed as described above with incubation times extended to 60 days. The results of this experiment demonstrated that neither pHP-1dl.2, with a 2-nt deletion in *env*, nor pHP-1dl.28, with a 28-nt deletion, generated detectable RCV (Table 2). The 28 nt *env* deletion in pHP-1dl.28 did not negatively affect virus titer which was consistently greater than 10^5 transducing units/per milliliter of vector supernatant 24 h following transfection of either TE671 or 293T cells (Table 3). To minimize the possibility of recovering env function, further deletions have been made in the *env* gene without affecting vector efficiency (unpublished).

RT and vector titer analyses of HP/TV versus wt HIV-1

To examine the pHP helper function and the pTV packaging efficiency, we first examined the viral reverse transcriptase (RT) production. HeLa cells were transfected with wt HIV_{NL4-3}, pHP-1, pHP-1dl.2, or pHP-1dl.28 and the culture supernatant was collected for RT assay 48 h later. Figure 2 shows that the levels of RT synthesis by the pHP-1-derived constructs were comparable to those observed for wt HIV-1; however, the vpr/tat-deleted pHP-VSVG produced only minute amounts of RT even when co-transfected with *tat* and *vpr*. Transfected cell lysates were also analyzed to see if the supernatant RT

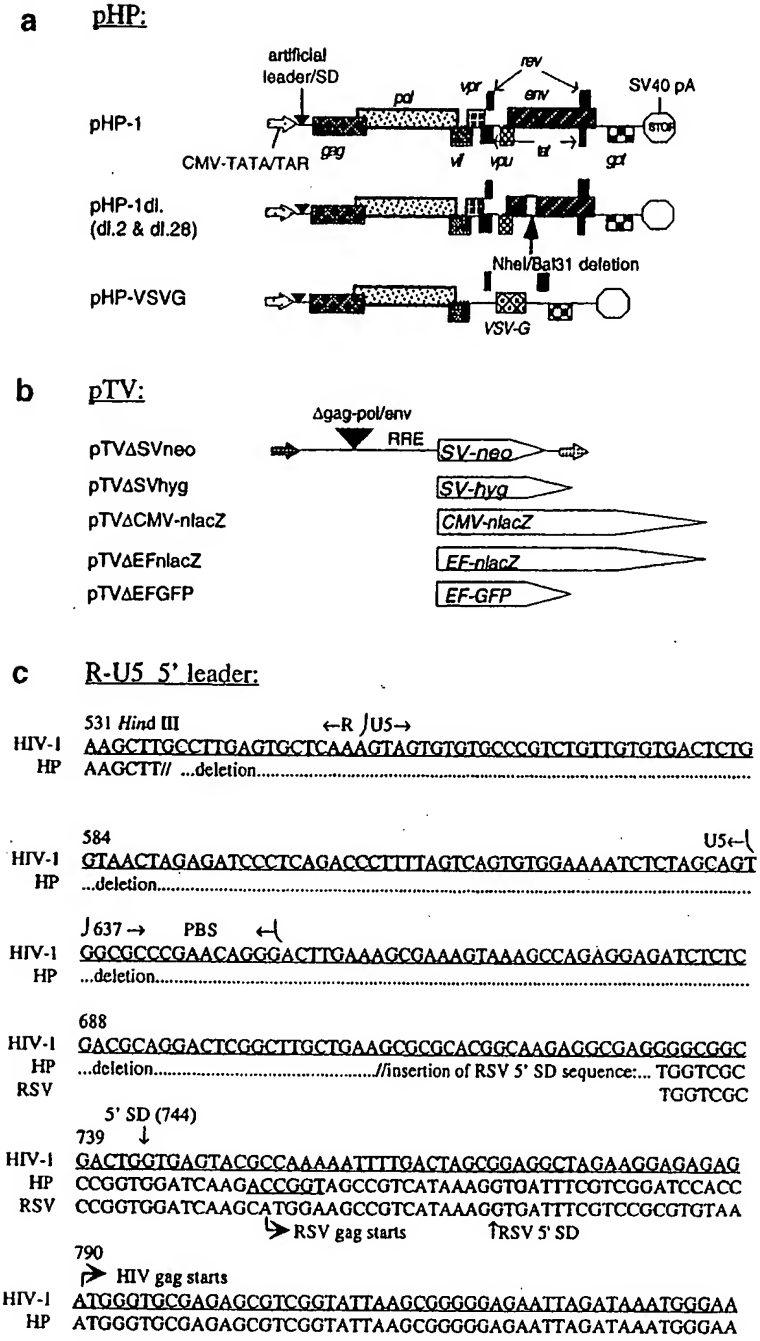

Figure 1 Illustration of recombinant HP/TV vectors. (a) Diagram of recombinant pHP packaging constructs. The parental construct pHP-1, the env-deleted pHP-1dl. and the recombinant pHP-VSVG packaging vectors are shown with corresponding HIV genes, mutations and a chimeric CMV-TAR enhancer/promoter. (b) Diagram of recombinant pTV transducing constructs. Five representative reporter gene constructs used in this study are illustrated. (c) pHP 5' leader sequences with a modified RSV splice donor site. The numbering of wt HIV-1 sequence is based on pNL4-3 (GeneBank Accession number M19921). A 59 nt leader sequence containing the RSV 5' SD site and mutations including the RSV gag AUG is illustrated.

activity correlated with intracellular Gag synthesis. The results showed that the amount of Gag protein produced by pHP-1 was similar to that produced by wt HIV-1 infected cells or transfected cells (lane 3 *versus* lanes 1 and 4, Figure 2 insert). Interestingly, although tat and/or vpr co-transfection did not markedly boost RT production of pHP-VSVG, it substantially increased the level of Gag synthesis as measured by Western blot (Figure 2, lanes 6 and 7 *versus* lane 5).

To compare helper function of pHP and wt HIV-1 further, TE671 or HeLa cells were co-transfected with a packaging vector (pHP-1, pHP-1dl.2 or wt pNL4-3), a transducing vector pTVΔCMVnlacZ, and the envelope construct pHEF-VSVG. RT activity and vector titer were determined and summarized in Table 4. The pHP packaging vectors produced RT at 30–40% of the wt level in TE671 cells but near wt level in HeLa cells. Interestingly, the vector titer assay indicated that HP/TV co-transfec-

Table 1 HP/TV vector transduction and RCV assays

pHP	pTV	Additional genes	Reporters[a]	RCV[b]
pHP-1	pTVΔSVneo	VSV-G	[illegible]	[illegible]
	pTVΔSVhyg	VSV-G	[illegible]	[illegible]
	pTVΔCMV-nlacZ	VSV-G	[illegible]	[illegible]
pHP-VSVG	pTVΔCMV-nlacZ	VSV-G, tat	[illegible]	[illegible]

[a]Reporter genes *neo* or *hyg* of pTVΔ were assayed by infecting HeLa cells with 50 μl and 100 μl of culture supernatant followed with G418 or hygromycin B selection 24 h after transduction or for *nlacZ* reporter gene by infecting HeLa cells with 20, 50 and 100 μl of culture supernatant and stained with X-gal 48 h later as described in Materials and methods. pHP-1/pTV co-transfection produced >[illegible]/ml vector and pHP-VSVG/pTV produced <[illegible]/ml vector; however, the titer was not precisely determined due to the generation of RCV.
[b]Replication-competent virus (RCV) assayed by co-culturing with MT4 cells for up to 30 days. RCV was detected by monitoring cytopathic effects on MT4 cells, RT and MAGI assays.

tions using either pHP-1 or pHP-1dl.2 produced two to three times more transducing units than the wt HIV_{NL4-3}/pTV co-transfection. The results of TE671 transfections further confirmed this observation (Table 5). Packaging constructs pHP-1, pHP-1dl.2 and pHP-1dl.28 produced vector titers three to seven times higher than that of the wt construct $HIV\text{-}1_{NL4-3}$. No RCV was detected from co-transfection of pTV with pHP-1dl.2 or pHP-1dl.28 using our sensitive RCV assay.

Transduction of growth-arrested and slow-dividing cells
To demonstrate that the HP/TV vectors are capable of transducing non-proliferating cells, HeLa cells were treated with mitomycin C to block cell division and then transduced with pTVΔCMV-nlacZ viral vector. The transduced cells were assayed for β-galactosidase activity 48 h later. The results indicated that the HP/TV vectors transduced normal dividing HeLa cells as efficiently as did growth-arrested HeLa cells (for examples, Figure 3a and b). This was further confirmed in numerous assays; we routinely use mitomycin C-treated cells and normal dividing cells for vector stock titration. The HP/TV vectors also efficiently transduced primary rat neuronal cultures (Figure 3d, e, f) and human neuronal stem cell-derived cultures (Figure 3g, h, i), both were post-mitotic, terminally differentiated cells. These experiments demonstrate that HP/TV vectors efficiently transduce growth-arrested or terminally differentiated nondividing cells. Interestingly, when human TE671 cells were transduced with high titer HP/TV vectors (pTVΔEFGFP), differentiated muscle cells expressing GFP reporter gene was observed (Figure 4). It is possible that the virion-associated Vpr was present at concentration high enough to induce differentiation of TE671 into muscle cells.

Human CD34 hematopoietic progenitor cells are a slow-dividing cell population which is known to be difficult to transduce with conventional retroviral vectors. To determine if HP/TV vectors can transduce this human cell type, freshly isolated human bone marrow-derived CD34 cells were transduced with two different constructs of lentiviral reporter vectors, pTVΔEFGFP and pTVΔEFnlacZ. The results showed that at MOI of 5–10, the HP/TV vectors successfully transduced human CD34 cells (Figure 5a to d, GFP; e to f, nlacZ). Comparison of HP/TV with MLV was also studied *in vivo* in rat muscle tissue. The result showed that intramuscular injections of the pTVΔCMV-nlacZ viral vector-produced nlacZ expressing muscle cells in 48 h; in contrast, injections with retroviral vectors did not produce reproducible positive results (not shown).

Short- and long-term analyses of lenti- versus retroviral transgene expression in human cells
The efficacy of gene therapy vectors is often judged by their transduction efficiency as well as long-term stability. To compare lentiviral and retroviral vectors directly, a long-term *in vitro* study was carried out. Three different human cell types, TE671, 293T and HepG2 cells, were transduced with either retroviral vector MFGnlacZ, that was produced from PA317 packaging cells and contained a MLV LTR-driven *nlacZ* gene, or lentiviral vector pTVΔCMVnlacZ, that was generated from co-transfection with pHP-1dl.28 and contained a CMV-IE promoter-driven *nlacZ* gene. About 10^5 transducing units of vector (approximately 1 MOI) were used for each transduction in a total of three rounds of transduction. Transduced cultures were grown until confluent (3–5 days), trypsinized, counted and plated into six-well culture plates.

Table 2 RCV assay following DNA co-transfection and long-term co-culture

Packaging construct	Pseudotyped envelope	Transducing vector	Additional gene	RCV assay (days after co-transfection)[a]		
				[illegible]	28	60
pNL4-3 (control)	pHEF-VSVG	pTVΔCMV-nlacZ		[illegible]	[illegible]	[illegible]
pHP-1	pHEF-VSVG	pTVΔCMV-nlacZ	tat	[illegible]	[illegible]	[illegible]
pHP-1dl.2	pHEF-VSVG	pTVΔCMV-nlacZ	tat	[illegible]	[illegible]	[illegible]
pHP-1dl.28	pHEF-VSVG	pTVΔCMV-nlacZ	tat	[illegible]	[illegible]	[illegible]

[a]The MT4 cells in the initial MT4 co-culture were transferred into a fresh MT4 culture on day [illegible] after co-culture and [illegible] days later the MT4 cells were directly immunostained with anti-HIV antiserum. [illegible]
[b]Results of rapid cell death [illegible] of MT4 cells [illegible] represent approximately [illegible] of the reporter [illegible] HeLa cells which were HIV positive after infection [illegible] of supernatant [illegible] RCV was detected [illegible]

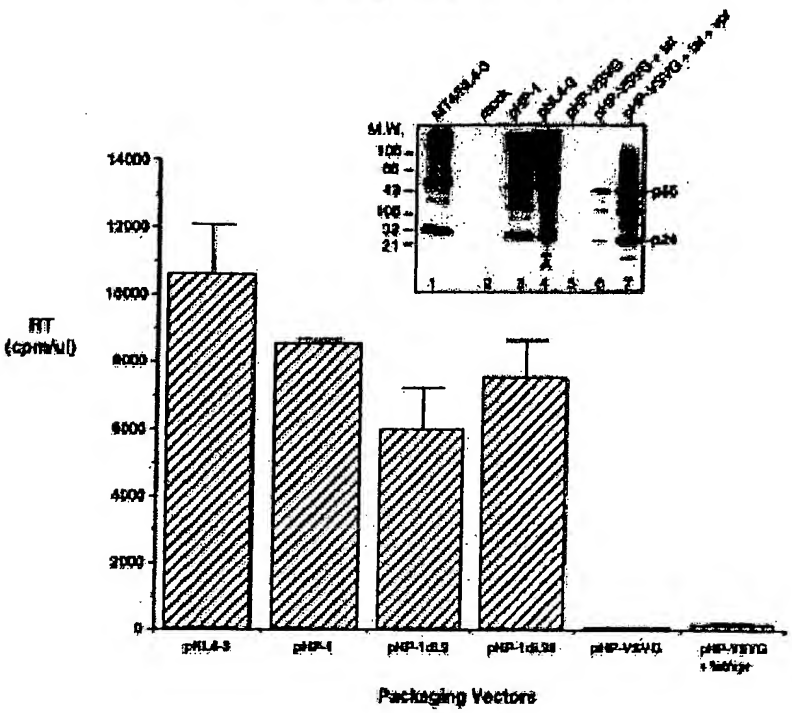

Figure 2 RT and protein syntheses of HP/TV versus wt HIV-1. Expression of cell-free RT was measured in duplicate using supernatant of cultures transfected with packaging vectors as indicated. The insert Western analysis was performed using the same amounts of cell lysates from HIV-1 infected (MT4/NL4-3, lane 1) or transfected cultures (lanes 2 to 7). Protein molecular weight markers and the predicted sizes for HIV-1 gag p55 and p24 are indicated.

Table 3 HP/TV vector production in TE671 versus 293T cells

Cell line	*pHP-*	*pTV-*	*p24 (ng/ml)*	*Titer (tu/ml)*
293T	pHP-1dl.28	pTVΔCMV-*nlacZ*	598	3.1×10^5
TE671	pHP-1dl.28	pTVΔCMV-*nlacZ*	573	2.3×10^5

Cells in T25 flasks were transfected with pHP DNA (12 μg), pHP-VSVG (7.5 μg), pTV (12 μg) and pCEP-tat (2 μg) by calcium phosphate co-precipitation method and the culture supernatant was harvested 48 h later for p24 ELISA and vector titration assay on TE671 cells as described in Materials and methods. tu, transducing units.

Twenty-four hours after plating, the cells were sampled for *lacZ* assay and the percentage of cells transduced was determined. The results of this short-term study showed that the lentiviral vectors transduced all three types of human cells three to 10 times more efficiently than did the MLV vectors (Table 6). For the long-term assays, the transduced cells were continuously propagated without selection. At different passage times, the percentage of *nlacZ*-expressing cells and the expression kinetics were determined (Figure 6). The results showed that the stability of transgene expression varied as a function of cell type. Expression of the HP/TV vector, which was driven by the CMV-IE promoter, decreased with time in all three cell types, whereas MLV transgene expression gradually decreased in 293T cells but not in TE671 or HepG2 cells.

Table 4 Comparison of vector titers between HP/TV and wt HIV-1/MLV co-transfections

Packaging construct	*Transducing vector*	*Additional gene*	*RT (cpm/ml × 10³)*	*Titer (tu/ml)*
pNL4-3	pTVΔGMV-*nlacZ*	VSV-G	10	1.0×10^6
pHP-1	pTVΔGMV-*nlacZ*	tat-VSV	8.7	1.2×10^6
pHP-1dl.9	pTVΔGMV-*nlacZ*	tat-VSV	9.4	1.2×10^6
pHP-1dl.38	pTVΔGMV-*nlacZ*	tat-VSV	9.4	1.2×10^6
pHP-VSV-G	pTVΔGMV-*nlacZ*		0.1	1.0×10^3
pHP-VSV-G + tat/rev	pTVΔGMV-*nlacZ*		0.1	1.0×10^3

Cells in T25 flasks were transfected with pHP DNA (12 μg), pTV (12 μg), pCEP-tat (2 μg) and pHP-VSV-G (7.5 μg) by calcium phosphate co-precipitation method and the titer was determined on TE671 cells as described in Materials and methods. Background activity of pHP plus pTV was not detected.

[illegible]

Packaging construct	Transducing vector	Additional gene	RT (cpm/µl)	Titer (/ml)	RCV
pNL4-3	pTVΔCMV-nlacZ	[illegible]	[illegible]	[illegible]	[illegible]
pHP-1	pTVΔCMV-nlacZ	tat	[illegible]	[illegible]	[illegible]
pHP-1dl2	pTVΔCMV-nlacZ	tat	[illegible]	[illegible]	[illegible]
pHP-1dl28	pTVΔCMV-nlacZ	tat	[illegible]	[illegible]	[illegible]

293T cells were co-transfected with plasmid DNA as indicated and vector titer and RCV were assayed as described in Materials and methods.
RT background activity of [illegible] cpm/µl was not subtracted.
RCV was assay by MT4 co-cultivation for 60 days and the survival MT4 cells were stained for HIV-1 antigens by immunohistochemical staining using an AIDS patient's serum.

Prolonged presence of lentiviral but not retroviral unintegrated proviral DNA in transduced cells

To determine if the HP/TV transgene was integrated, extrachromosomal (Hirt) and chromosomal DNA was harvested from the lentiviral vector-transduced cells and analyzed by Southern blotting. Figure 7a shows that nlacZ sequence was detected at passage 7 in the chromosomal DNA of TE671 and HepG2 cells (lanes 4 and 5), but not in the Hirt preparations (lanes 2 and 3). To investigate further the causes of the reduction of transgene expression, we compared the chromosomal and Hirt DNA harvested from early and late passages of the transduced culture by Southern analysis. The results of these analyses showed that similar amounts of nlacZ DNA were present in early (passage 3) and late passages (passage 40) of 293T (Figure 7b, lanes 4 and 5, respectively) and HepG2 cells (passage 6 *versus* passage 49, Figure 7c), even though the expression kinetics indicated that nlacZ expression in these cells gradually decreased with time. In contrast, in TE671 cells the integrated DNA was lost in late passages (Figure 6b, lane 3 passage 40, *versus* lane 2 passage 4), a result consistent with the observed decrease in nlacZ expression with time.

To see if the presence of unintegrated proviral DNA in the short- and long-term cultures could explain the transient high expression phenotype of lentiviral vectors, a more sensitive assay was performed. Hirt DNA was harvested from early and late passages of cells transduced with either HIV or MLV vectors and analyzed by PCR using nested primers that specifically amplify one- or two-LTR unintegrated proviral DNA circles. One-LTR proviral DNA was detected in all three HP/TV vector transduced cultures from early passages (Figure 8a, TE671, 293T and HepG2 passage 3–5, lanes 3, 5 and 7, respectively) but not from late passages (passage 36–44, lanes 4, 6 and 8). Further amplification of the first round nested PCR products did not reveal the presence of lentiviral proviral DNA in the late passages of cells (not shown). In contrast, MLV proviral DNA was not detected either from early or from late passages of transduced TE671, HepG2 or 293T cells via a sensitive nested PCR assay using two sets of nested primers (passage 3–39 of MLV transduced cells, Figure 8b).

Discussion

The ability of lentiviruses to infect nondividing cells such as macrophages and neurons makes them good candidates for use as gene transfer tools. However, the complicated genome organization and regulation of viral gene expression, as well as the concerns of possible spread of AIDS with HIV-derived vectors, have hindered the wide dissemination of lentiviral vector technology. Using previously established attenuated HIV-1 constructs,[10,11] we have successfully generated a recombinant lentiviral vector gene transfer system that is efficient and safe. Efficient synthesis of HIV-1 Gag-Pol requires Tat activation of LTR and the interaction of Rev–RRE to mediate nuclear export of mRNA, whereas the accessory gene functions of vif, vpr, vpu and nef have been shown to be dispensable for viral replication, as well as for vector function in tissue culture.[12,13] Both *tat* and *rev* genes are functional in the gag-pol packaging construct pHP. In pHP, several *cis*-elements essential to viral replication have been deleted, including both the 5′ and the 3′ LTRs, the 3′ PPT and the entire 5′ leader sequences except for TAR. A 59 nt artificial RSV splice donor sequence has been inserted into pHP to support tat and rev mRNA splicing. As illustrated in Figure 1c, the RSV gag AUG is located in the 59 nt artificial leader sequence 5′ to the RSV SD site. To prevent interference with the use of the downstream HIV gag AUG, the RSV gag AUG was mutated in pHP. Although it is not clear if mutation of the RSV gag AUG could affect the RSV SD function, the expression of functional *tat* and *rev* genes by pHP suggests that it did not affect the vector function.

Compared with wt HIV-1, the changes made in pHP had little effect on viral RT synthesis, nor did it diminish the vector titer. Interestingly, although wt HIV-1 exhibited higher RT activity than pHP when co-transfected with a pTV vector, wt HIV-1 produced fewer infectious vectors than pHP, possibly due to the interference of wt genome with vector genome packaging. In fact, the HP/TV vector system consistently produced three to five times more vector than did the wt HIV/TV co-transfections. Western blot analyses showed that HP produced Gag at levels similar to that of wild-type HIV-1. This result is consistent with the results of our previous studies in which we found that the CMV-TAR chimeric promoter exhibits high transcriptional activity and remains Tat responsive.[14]

We examined RCV production via recombination of HP/TV by testing *env*-deleted HP constructs in co-transfection experiments. By coupling the co-culture method with immunostaining, we were able to detect very low amounts of RCV. Using this assay, RCV was easily detected in cultures co-transfected with the original pHP-

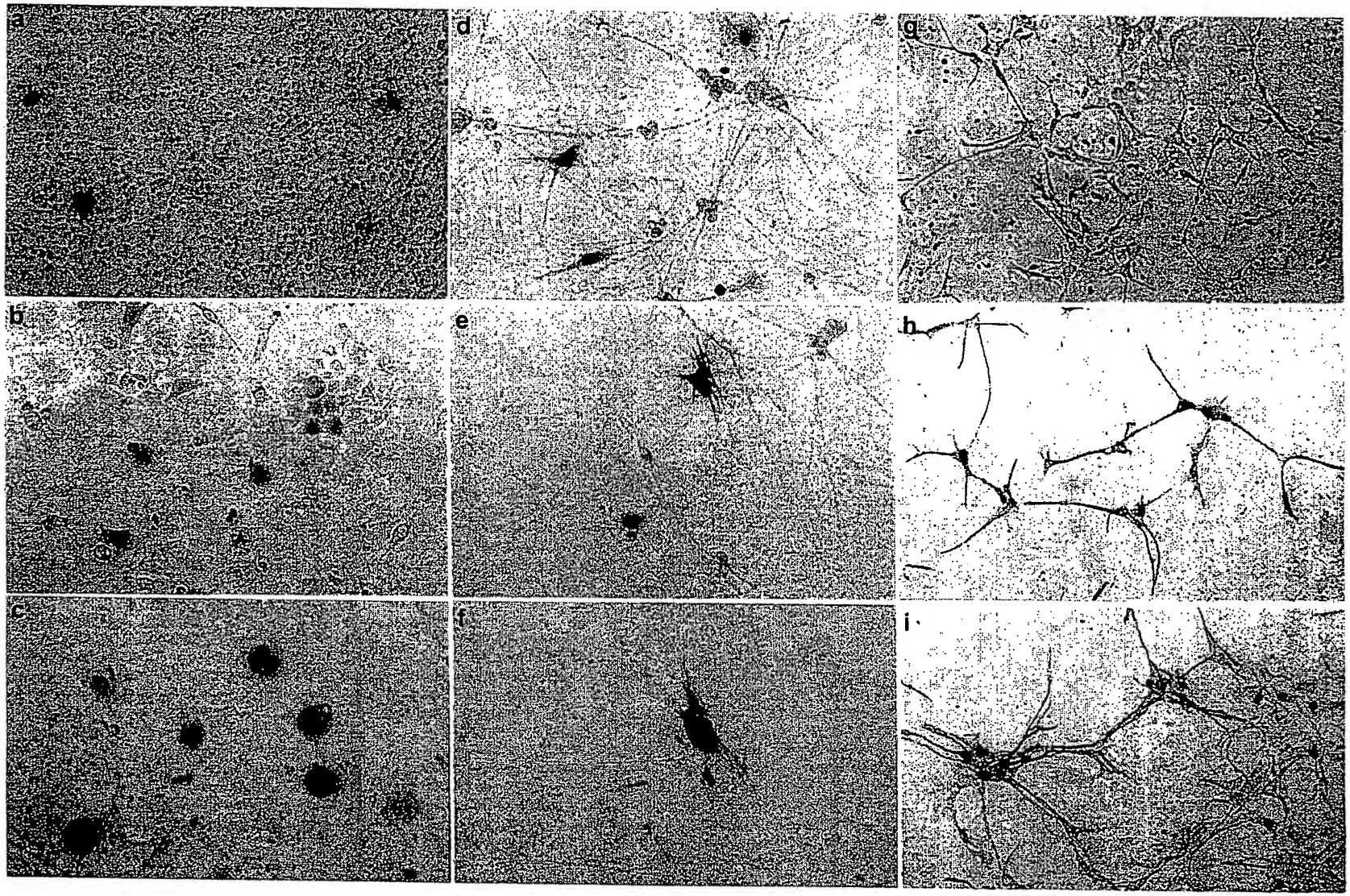

Figure 3 *Lentiviral vector transduction of growth-arrested or terminally differentiated cells. (a) Dividing HeLa cells; (b and c) mitomycin C-treated HeLa cells (×10 and ×20 object magnification, respectively); (d, e and f) primary rat neurons; (g, h and i) human neuronal stem cell-differentiated cells. HeLa cells were transduced with VSV-G pseudotyped pTVΔCMV-nlacZ and analyzed for nlacZ expression 48–72 h later. Rat neurons and human neuronal stem-cell-derived culture were growth arrested, transduced, cultured for 4–5 days and analyzed for nlacZ expression as described in Materials and methods.*

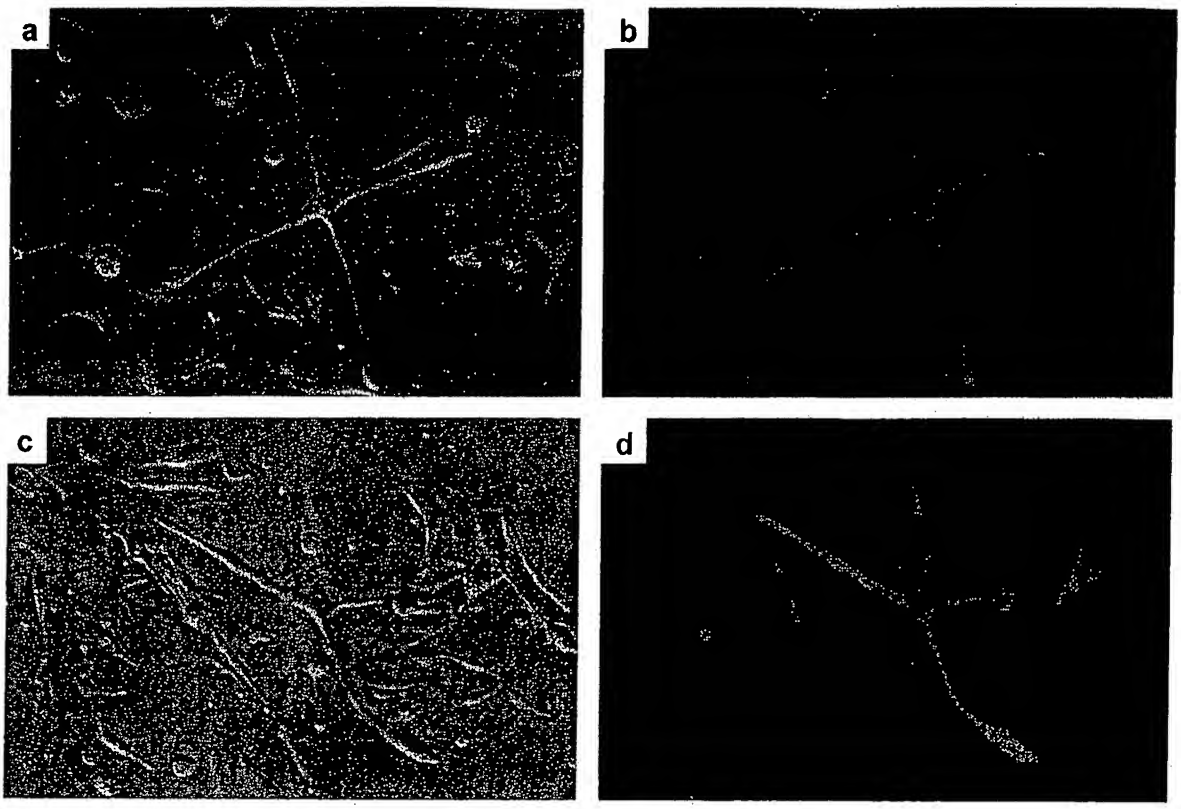

Figure 4 Lentiviral transduction and differentiation of human rhabdomyosarcoma cells. Human TE671 cells were transduced with lentiviral vector pTVΔEFGFP three times (3–5 MOI) and incubated for 4–5 days. The GFP gene expression was detected directly under a Zeiss Axiovert 25 inverted fluorescent microscope with ×10 Fluar objective lens (Carl Zeiss, Thornwood, NY, USA). (a and c) phast-contrast photographs (10× 10); (b and d) green fluorescent cells corresponding to a and c.

1 construct which contains a wild type *env* gene, but not in cultures co-transfected with pHP-1dl.2 which has a 2-nt deletion between the C5 and the C6 domains of env gp120. The absence of RCV production in these cultures was likely due to the lack of *env* function rather than the lack of recombination because a recombinant virus lacking *nef*, but containing a reporter gene, could still be generated via a double genetic crossover between pHP and pTV. Additional mutations had been introduced in pHP and pTV to eliminate homologous recombination which effectively eliminated the possibility of RCV production (Cui *et al* and Iwakuma *et al*, submitted).

In a previous study, the transduction efficiencies of an HIV-1-based vector and an MLV-based vector have been compared in both proliferating and growth-arrested HeLa cells and 208F rat fibroblasts. The results show that the HIV-based vectors infect G1/S and G2 arrested HeLa cells and G0 arrested 208F cells more efficiently than the MLV-based vector.[8] In this study, we showed that the HP/TV vector transduced growth-arrested, terminally differentiated or slow-dividing cells efficiently. We have also demonstrated efficient transgene expression *in vivo* in rat muscles after injection with the HP/TV vectors. Retroviral vectors failed to transduce these cell types. In addition, we tested three different human cell lines, TE671, 293T and HepG2 and compared short-term and long-term transduction using both an HIV-1-based HP/TV vector and an MLV-based MFG vector. Again, our results showed that the HP/TV vector expressed the bacterial *nlacZ* transgene at higher efficiencies than the MFG vector in all three cell lines, but that expression of the lentivirally transduced *nlacZ* gene gradually decreased after multiple passages. After more than 35 passages, the expression level of the HP/TV *nlacZ* transgene was similar to that observed for the MLV *nlacZ* transgene. Southern analyses of TE671 genomic DNA suggested that the HP/TV *nlacZ* transgene was integrated into the target cells, but was lost from the cultures upon repeated passages. Southern analyses of 293T and HepG2 genomic DNA indicated that the transgene was still present in these cells even after 49 passages, a result suggesting that the decrease of HP/TV *nlacZ* transgene expression may have been due to loss of promoter activity.

Hirt DNA analyses indicated that unintegrated lentiviral proviral DNA could persist in transduced cells for more than four to five passages, but that after 40 passages it could not be detected in any of the three cell types examined. In contrast, no MLV-derived unintegrated proviral DNA was detected as early as passage 3 in all the cell types examined. Together, these data suggest that lentiviral vectors are more efficient than MLV vectors possibly attributable to the prolonged presence of unintegrated lentiviral proviral DNA that may drive lentiviral transgene expression. Stevenson *et al*[15] reported that unintegrated HIV-1 DNA can serve as a template for HIV-1 antigen synthesis, whereas Sakai *et al*[16] reported opposite results with an HIV-1 integrase mutant. Our

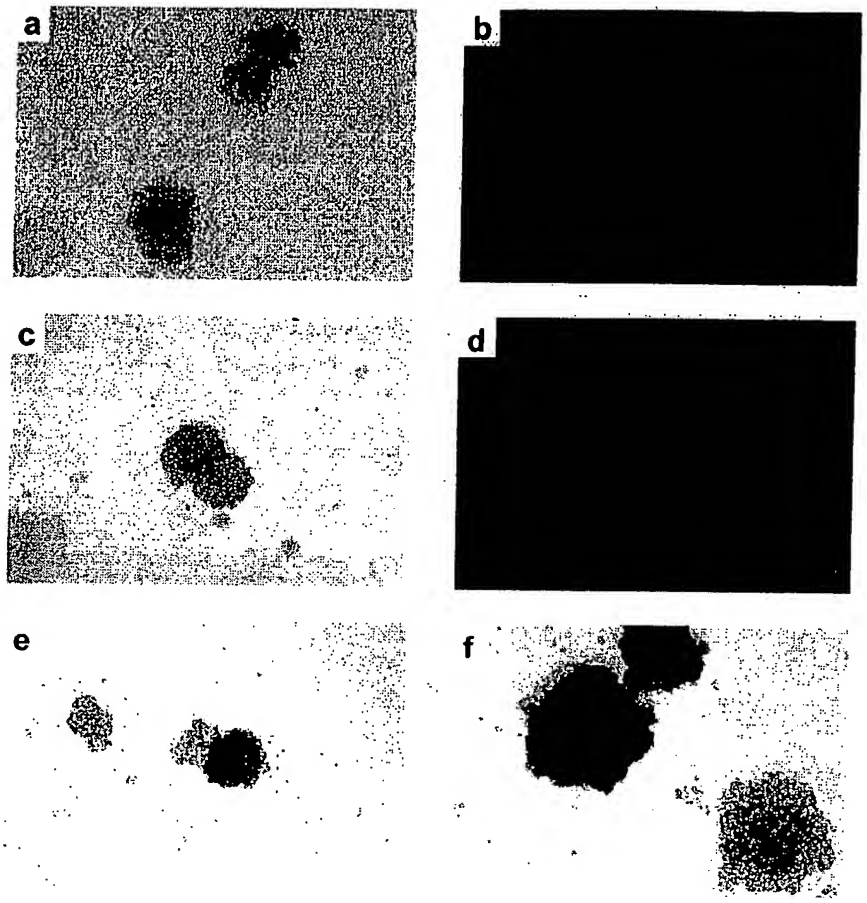

Figure 5 Lentiviral transduction of human CD34⁺ hematopoietic progenitor cells. Human CD34⁺ cells were collected and transduced with pTVΔEFGFP (a to d) or pTVΔEFnlacZ (e and f) at approximately 5–10 MOI as described in Materials and methods. The transduced cells were plated in semi-solid methylcellulose culture for 2–3 weeks. The GFP transgene expression (b and d) was observed under a fluorescent microscope as described in Figure 4 legend. (a and c) The same fields viewed under microscope as b and d, respectively, with a regular instead of a fluorescent light source. The nlacZ transgene expression (e and f) was detected following X-gal staining as described in Materials and methods at 37°C after 2–3 days.

Table 6 Short-term transduction efficiencies of lentiviral vector (pTVΔCMVnlacZ) versus MLV vector (pMFGnlacZ) in three different human cell lines

Human cells	HIV vector	MLV vector
	(% transduced cells after 1 passage)	
U6671 (rhabdomyosarcoma)	62 ± 4	26 ± 4
293T (kidney carcinoma)	46 ± 2	7 ± 0.5
Hep G2 (hepatoma)	29 ± 6	2 ± 3

Values given are % transduced cells after 1 passage. Cells were transduced three times and propagated once before staining for β-galactosidase expression.

data show that high level lentiviral vector transgene expression coincides with the presence of unintegrated proviral DNA which is consistent with Stevenson's observation. It is conceivable that the internal CMV-IE promoter in the unintegrated HP/TV proviral DNA is active but the 5′ LTR promoter is not because the latter requires Tat transactivation.

We have observed differentiation of TE671 cells into muscle cells after transduction with vpr+ but not with vpr– HP/TV vectors (Figure 4 and data not shown). Thus, it is also possible that the loss of integrated pTV gene in TE671 culture was due to the disappearance of transduced and differentiated TE671 cells with time. On the other hand, pTV contains a CMV-IE enhancer/promoter which has been shown to be inactivated frequently after integration.[17,18] Promoter inactivation could therefore have contributed to loss of HP/TV gene expression. Interestingly, in a separate study, we observed increased transgene expression with time, even after passage 30, in TE671 cells transduced with lentiviral vector carrying a human growth hormone gene under control of different internal promoters, CMV-IE and human elongation factor 1α promoters (Iwakuma and Chang, unpublished). Thus, the expression phenotype of a transgene is strongly influenced by the characteristics of the transduced cell, the internal promoter as well as the transgene itself. Current research efforts are directed toward further modification of the HP/TV vector system

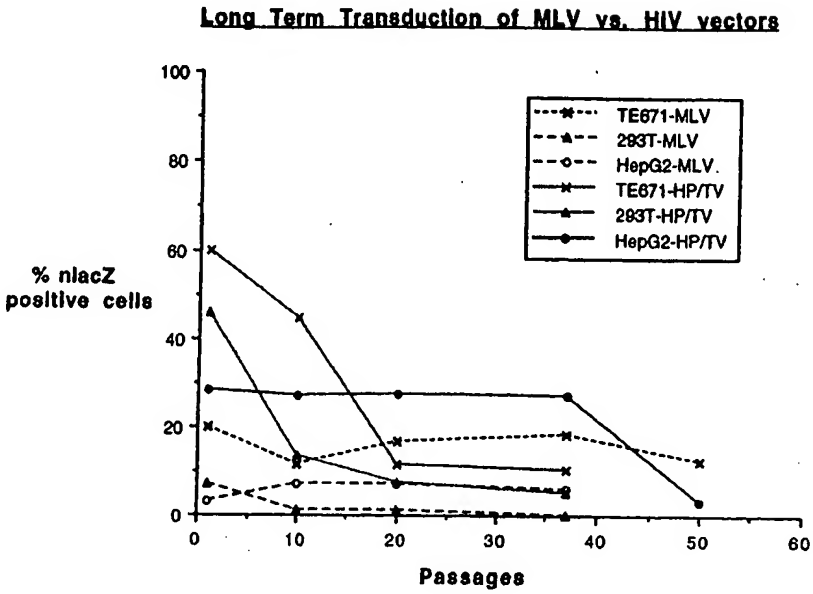

Figure 6 Kinetics of retro- and lentiviral transgene expression in three different human cell lines. Cells were transduced with 10^5 tu of pTVΔCMV-nlacZ or pMFGnlacZ and propagated for long-term study. At different passage times as indicated, cells were collected and stained for β-galactosidase activity to determine the percentage of positive cells.

to improve both the safety and the long-term efficacy of this lentiviral vector system.

Materials and methods

Cells and animals

HeLa (human cervical carcinoma) and HepG2 (human hepatoma) cells were obtained from ATCC (Rockville, MD, USA). TE671 (human rhabdomyosarcoma) and 293T (transformed human primary embryonal kidney) cells were kindly provided by Dr Takeuchi (Chester Beatty Laboratories, obtained from ECACC, UK) and Dr H Goldstein (Albert Einstein College of Medicine, NY, USA), respectively. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco Canada), penicillin and streptomycin, and mycoplasma contamination was monitored periodically and eliminated if found.[19] Sprague–Dawley rats (180–200 g of body weight) were purchased from the Health Science and Laboratory Animal Service (HSLAS) at the University of Alberta. The HP/TV vector system has gone through extensive RCV testing and has been approved for use in a biosafety level II laboratory employing a level III standard operating protocol. The level III protocol was approved by the Biosafety Committees of the University of Alberta and the University of Florida. The lentiviral vector tissue culture and animal studies were performed in a level II+/III laboratory using protocols approved by the Animal Welfare Committees and the Biosafety Committees at the University of Alberta and at the University of Florida.

Plasmid construction

pHP-1 was generated as follows. First, a recombinant enhancer/promoter CMV-TATA-TAR fragment was isolated from dl.kB/Sp1-CMV-TATA-TAR HIV[11] by *Bbr*pI–*Hin*dIII digestion. This fragment was cloned into the *Eco*RV–*Bam*HI site of pSP72 (Promega, Madison, WI, USA) using a linker possessing *Hin*dIII and *Bam*HI cohesive sites. The pSP72 vector contains a modified gag AUG located within a favored eukaryotic translation initiation sequence context (-CCACCATG-), and a major splice donor site derived from Rous sarcoma virus (RSV) containing a mutated RSV gag AUG.[20] The linkers used in this construct were created by annealing the following oligonucleotides: 5′-AGC TTG GTC GCC CGG TGG ATC AAG ACC GGT AGC CGT CAT AAA GGT GAT TTC GTC G-3′ and 5′-GAT CCG ACG AAA TCA CCT TTA TGA CGG CTA CCG GTC TTG ATC CAC CGG GCG ACC A-3′. Secondly, the gag coding sequence for the pHP-1 construct was amplified from the HIV-1 molecular clone pNL4-3 using PCR and the following primers which encompass the gag gene: sense, 5′-CGG GAT CCA CCA TGG GTG CGA GAG CGT C; antisense, 5′-ATC CTA TTT GTT CCT GAA GG. The PCR product was digested with *Bam*HI-*Sph*I and ligated to the chimeric CMV-TAR promoter. Thirdly, a subclone of pHP-dl.pA without the polyadenylation signal was made by ligating together the 1112 bp *Hpa*I–*Sph*I fragment isolated from the chimeric CMV-TAR-gag construct, the 7922 bp *Sph*I–*Xho*I fragment isolated from pNLgpt and a plasmid vector backbone obtained by digesting pBS-KS(–) (Stratagene, La Jolla, CA, USA) with *Eco*RV and *Xho*I. pNLgpt was generated by cloning the *E. coli* xanthine-guanine phosphoribosyltransferase (gpt) gene from pMSG (Pharmacia, Uppsala, Sweden) into the nef open reading frame (ORF) of pNL4-3 between the nef AUG and the *Xho*I site. Finally, pHP-1 was generated by ligating a 422 bp *Xho*I–*Pst*I digested SV40 polyadenylation signal obtained from pREP9 (Invitrogen, Carlsbad, CA, USA) into pHP-dl.pA.

The pHP-VSVG vector was generated by ligating the following four DNA fragments together: (1) the *Not*I-*Eco*RI fragment of pHP-1 containing the chimeric CMV-TATA-TAR-gag-pol sequence; (2) a fragment generated from HIV-1 possessing a deletion in *vpr* and *tat* that was

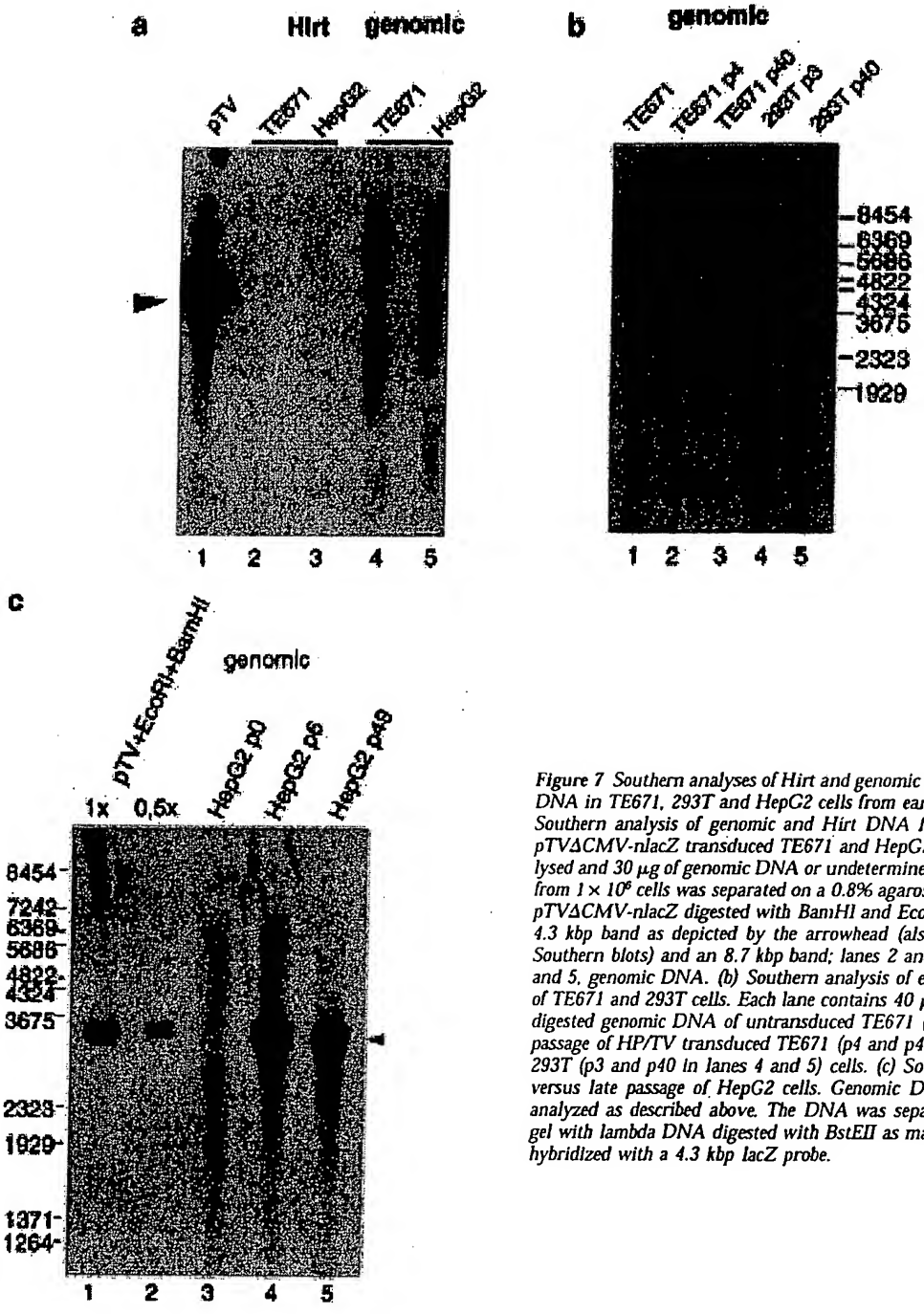

Figure 7 Southern analyses of Hirt and genomic lentiviral vector proviral DNA in TE671, 293T and HepG2 cells from early and late passages. (a) Southern analysis of genomic and Hirt DNA from early passage cells. pTVΔCMV-nlacZ transduced TE671 and HepG2 cells at passage 7 were lysed and 30 μg of genomic DNA or undetermined amounts of Hirt DNA from 1 × 10⁶ cells was separated on a 0.8% agarose gel; lane 1 is a control pTVΔCMV-nlacZ digested with BamHI and EcoRI, which gave rise to a 4.3 kbp band as depicted by the arrowhead (also used as the probe for Southern blots) and an 8.7 kbp band; lanes 2 and 3, Hirt DNA; lanes 4 and 5, genomic DNA. (b) Southern analysis of early versus late passage of TE671 and 293T cells. Each lane contains 40 μg of BamHI and EcoRI digested genomic DNA of untransduced TE671 (lane 1), or early or late passage of HP/TV transduced TE671 (p4 and p40 in lanes 2 and 3) and 293T (p3 and p40 in lanes 4 and 5) cells. (c) Southern analysis of early versus late passage of HepG2 cells. Genomic DNA was processed and analyzed as described above. The DNA was separated on a 1% agarose gel with lambda DNA digested with BstEII as marker and the blots were hybridized with a 4.3 kbp lacZ probe.

introduced by PCR mutagenesis using the primers, sense-TAA GAA TTC TAG TAG GTT GCT TTC ATT GCC and antisense-CTT CTC CTT CAV TCT CGA GTG ATC ACT GTC TTC TGC TCT TTC- (encompassing env AUG where a new *Xho*I and a *Bcl*I site were created) as previously described;[10] (3) a *Sal*I–*Xba*I VSV-G gene fragment obtained from pBS-VSV-G (kindly provided by Tom Hobman at University of Alberta); and (4) the *Nhe*I–*Not*I fragment of pHP-1 containing the 3′ env gp120-gpt-SVpA and plasmid backbone. The differences between the final pHP-VSVG construct and the pHP-1 vector are that the region between the 3′ end of the *vpr* coding region and the 5′ end of the *tat* first exon has been deleted and a portion of the HIV-1 gp120 *env* gene has been substituted with *VSV-G* gene in the pHP-VSVG construct.

To generate the env-deleted pHP-1 constructs, pHP-1 was digested with *Nhe*I and treated with Bal31 exonuclease for 1, 2 or 5 min. The digest products were self-ligated following treatment with T4 DNA polymerase. Competent *E. coli* DH5α were transformed with the self-ligated plasmid DNA and from a pool of more than 48 deletion mutants, two clones (pHP-1dl.2 and pHP-1dl.28) were selected, sequenced and used in this study.

pTVΔSVneo and pTVΔSVhyg were generated by delet-

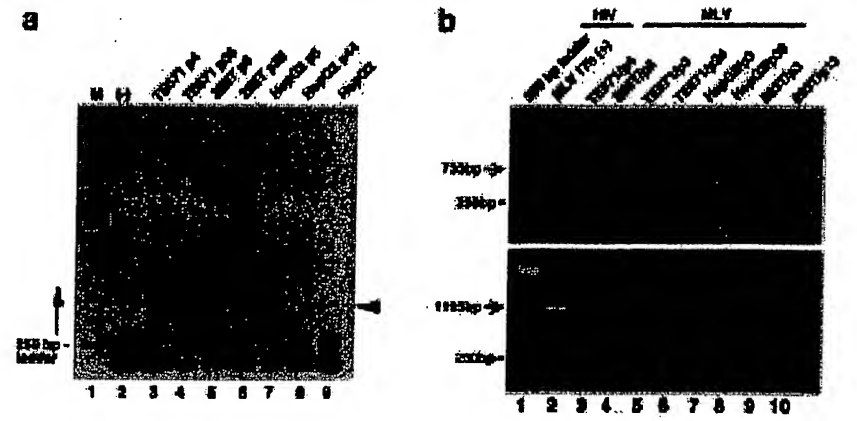

Figure 8 PCR analyses of unintegrated proviral DNA of HIV and MLV vector transduced cells. (a) PCR analysis of unintegrated lentiviral proviral DNA in early and late passage of TE671, 293T and HepG2 cells. Hirt DNA was harvested from cells of early and late passage as indicated and amplified by PCR using two sets of nested primers specific for unintegrated one- and two-LTR circles of lentiviral vectors as described in Materials and methods and analyzed on a 1% agarose gel. The arrowhead points to the amplified proviral one-LTR circular DNA. Nonspecific PCR products appeared in many of the Hirt DNA preparations upon PCR amplification which did not correlate with the presence of the 715 bp one-LTR PCR product. The presence of lentiviral unintegrated proviral DNA was confirmed by generating a longer PCR product using a different primer situated between the two nested 3' primers and further verified by Southern blot analysis (not shown). TE671 passage 36 (lane 4) was confirmed to be negative upon repetitive PCR analyses. (b) PCR analysis of unintegrated MLV proviral DNA in early and late passage of TE671, 293T and HepG2 cells. Two different sets of primers were used as described in Materials and methods and the corresponding products of 733 bp and 1195 bp are depicted. Hirt DNA from TE671 cells transduced with MLV vector for 17 h was used as positive control (lane 2). Hirt DNA from lentiviral vector transduced TE671 and 293T cells (HIV, lanes 3 and 4) was used as negative control. Molecular weight markers are shown in lane 1 as 250 bp DNA ladders.

ing gag-pol and env sequences starting from the *Spe*I site in the middle of the gag ORF extending to the *Nhe*I site in the middle of the env ORF of pNLSVneo and pNLSVhyg, respectively. pNLSVneo and pNLSVhyg were generated by inserting a SV40 promoter-driven neomycin- or hygromycin B-resistant gene between the nef AUG, where a *Hin*dIII site had been generated by site-specific mutagenesis and the unique *Xho*I site in nef in pNL4-3. pTVΔCMVnlacZ was made by inserting a *Sal*I–*Kpn*I digested CMV-nlacZ fragment, derived from pcDNA3-nlacZ, into *Xho*I–*Kpn*I digested pTVΔSVneo. The nuclear *lacZ* gene was generated by fusing a nuclear localization signal of SV40 large T antigen to the N-terminus of the bacterial *lacZ* gene that was obtained from the pBlueBac-HisA vector (Invitrogen) using PCR mutagenesis. The oligonucleotide primer 5'- CCC GGG TCT AGA AGC TTC CAC CAT GCC TAA GAA GAA ACG AAA GAT CGA TCC CGT CGT TTT ACA ACG TCG-3', which contains a favored eukaryotic translation initiation codon (underlined), was used in the PCR procedure. pTVΔEFGFP was generated by replacing the CMVnlacZ gene of pTVΔCMVnlacZ with the human elongation factor 1α promoter of pHEF kindly provided by D Denny plus a GFP reporter gene. pTVΔEFnlacZ was then made by replacing the GFP gene of pTVΔEFGFP with *nlacZ* gene. The VSV-G envelope expression plasmid pHEF-VSVG was constructed by inserting a PCR-amplified VSV-G fragment containing a favored translational initiation codon into the *Eco*RI site of pHEF. The mutagenesis site and flanking sequences of all the constructs were confirmed by DNA sequencing.

DNA transfection

The MLV-derived vectors were generated by transfecting the packaging cell line PA317 with pMFGnlacZ which contains the same recombinant *nlacZ* gene as the one cloned into pTVΔCMVnlacZ. The HP/TV vectors were generated by co-transfecting HeLa or TE671 cells with the pHP, pTV and pHEF-VSVG plasmids. A modified calcium phosphate DNA transfection protocol was performed as previously described.[10] The transfection efficiency, normally ranging from 50 to 90%, was determined by X-gal staining or by a radioimmunoassay for human growth hormone when the XGH5 plasmid was included in the transfection procedure (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). The transfected cells normally produce retro- or lentiviral vectors with titers ranging from 10^5–10^6 transducing units (tu) per ml. The VSV-G pseudotyped vectors were routinely concentrated 30–50 times by centrifugation in a table-top microfuge (21 000 *g*) for 2.5–3 h at room temperature and resuspended by vortexing in a thermomixer at room temperature for 2–3 h.

Vector transduction and titration

Virus supernatants were harvested 24, 48 and 72 h following addition of DNA by low speed centrifugation (1200 *g* for 5 min) or by filtration using a 0.45 μm low-protein binding filter to remove cell debris from transfected culture. No reduction in titer was observed between 24–48 h, but a one-log reduction in titer was frequently observed in harvests carried out at 72 h. The supernatants were aliquoted and stored at −80°C until use. Retroviral vector was titered on HeLa or TE671 cells and lentiviral vector was titrated on mitomycin-C-treated TE671 cells (5 μg/ml for 2.5 h). To titer the vector, cells were infected with diluted virus stocks at low MOI in a small volume of growth medium containing polybrene (8 μg/ml). Cultures were grown for 3–4 h, supplemented with additional growth medium and then incubated for a final period of 48 h before staining. At least two different dilutions were examined for each titration sample. To detect the transduced cells, the culture was washed twice with phosphate buffered saline (PBS) and fixed at room temperature with 1% formaldehyde and 0.2% glutaraldehyde (Sigma, St Louis, MO, USA) in PBS for 5 min.

After three more washes with PBS, the cells were incubated at 37°C in PBS or distilled water containing 4 mM K-ferrocyanide, 4 mM K-ferricyanide, 2 mM $MgCl_2$ and 0.4 mg/ml X-gal overnight. The CD34-derived cell colonies were stained under similar condition with a modified X-gal solution with adjusted pH at 8.5 to eliminate background mammalian β-galactosidase activity. The transduced cells stained with X-gal were examined with an inverted microscope. The high background β-galactosidase activity in HepG2 cells, primary cultures and tissues can also be eliminated by increasing the pH of the incubation buffer.

Mitomycin-C treatment

To arrest cell cycle, TE671 or HeLa cells were treated with mitomycin-C at 10 μg/ml for 4 h, trypsinized and plated into a six-well culture plate. Cell cycle arrest was monitored by propidium iodide staining and FACS analysis. The cells were transduced with HP-TV nlacZ vector in the presence of 8 μg/ml polybrene in a volume of 0.5 ml for 2–3 h and fed with growth media. After 48 h, the nuclear *lacZ* expression was detected by X-gal staining as described above.

Primary culture and in vivo transduction

The rat neuronal cells, kindly provided by Dr Campenot from University of Alberta, were isolated from rat brains according to the protocol of Ure *et al*,[21] and grown in culture media containing 10 μM cytosine arabinoside (Sigma) which inhibited cell proliferation. The human neurons and astrocytes were obtained from differentiated embryonic neural stem cells prepared and provided by Dr M Arcellana-Panlilio at Neurospheres (Calgary, Alberta, Canada). These cells were infected with the HP-TV vectors carrying the *nlacZ* reporter gene as described above. Briefly, cells were incubated in culture media containing the HP-TV vector. After a 2 h incubation, conditioned media (from cultured cells) was added to the cultures and they were incubated for an additional 5-day period. Following incubation, the infected cells were fixed and processed for X-gal staining as described above. *In vivo* transduction of rat muscle was performed using a 25-gauge insulin syringe. pTVΔCMVnlacZ vector, $2–5 \times 10^5$ tu/ml, was prepared from transfected TE671 and used for intramuscular injection. Each injection site received 200 μl of unconcentrated vector or 20 μl of 30–50 × concentrated vector.

Human CD34 cells were obtained from human bone marrow or mobilized peripheral blood cells collected after obtained informed consent from patients treated with G-CSF to mobilize bone marrow derived $CD34^+$ cells. Enrichment for $CD34^+$ cells was performed using an anti-CD34 antibody affinity column (CellPro, Bothel, WA, USA). The $CD34^+$ cells were washed two to three times with RPMI medium containing 10% FBS and resuspended in RPMI + 10% FBX supplemented with 50 ng/ml human flt3 ligand, 50 ng/ml human c-kit ligand and 50 ng/ml human IL-3 at $1–5 \times 10^5$ cells per 100 μl. The $CD34^+$ cells was transduced two to three times with pTV vectors containing the *nlacZ* or *GFP* reporter genes with 8 μg/ml polybrene for 3–4 h each time. The transduced $CD34^+$ cells were maintained in RPMI medium supplemented with growth factors for 1–4 days before they were plated in semi-solid methylcellulose colony assay. After 2–3 weeks in culture, the expression of transduced *nlacZ* or *GFP* genes was assayed by X-gal colorimetric staining or observed under an inverted fluorescent microscope.

RT assay and p24 ELISA

RT assay was performed as previously described.[11] p24 ELISA was performed using reagent kits purchased from SAIC (Frederick, MD, USA) and the protocols provided therein.

RCV assay

Assays for the detection of RCV included: (1) co-cultivation with a sensitive cell line such as MT4, AA2 or PBLs; (2) MAGI cell assay which relies on Tat transactivation of an integrated LTR-nlacZ gene in HeLaCD4 cells;[22] and (3) immunohistochemical staining for the detection of Gag p24 antigen expression at single cell level.[10] In the long-term co-culture RCV assay, MT4 cells were added to the HP/TV transfected cells 48 h after transfection. Fresh medium was added to the cultures every 3 days at which time two-thirds of the culture media was discarded. Fresh MT4 cells were added to the cultures once a week. Syncytium formation was observed under an inverted microscope. At different time-points, MT4 cells in the co-culture were collected and immunostained using a monoclonal anti-p24 antibody for the detection of HIV-1 Gag antigen as described previously.[10]

Genomic and Hirt DNA preparation and Southern and PCR analyses

A modified protocol was used which allowed simultaneous preparation of genomic and Hirt DNA.[23] Briefly, cells were washed three times with PBS and resuspended in 250 μl 25 mM Tris-HCl pH 8.0 buffer containing 50 mM glucose and 10 mM EDTA. The resuspended cells were incubated at room temperature for 5 min and then lysed in 200 μl lysis buffer containing 200 mM NaOH and 1% SDS on ice for 5 min. The lysate was neutralized by adding 150 μl potassium acetate (5 M, pH 4.8). Cell debris and chromosomal DNA (in pellet) were removed by centrifugation at 10 000 *g* for 5 min. The supernatant containing the Hirt DNA was loaded on to a QIAprep Spin Column (Qiagen, Santa Clarita, CA, USA) and centrifuged for 1 min. Columns were washed to remove residual endonucleases and salts, and the DNA was eluted with 100 μl distilled water (75°C) by centrifugation at 10 000 *g* for 1 min. The pellet containing the genomic DNA was processed using a Qiagen genomic DNA harvesting kit starting from the proteinase digestion step according to the manufacturer's instructions.

Southern analysis was performed using standard protocols as described by Maniatis *et al*[24] and a modified hybridization procedure as described previously.[10] PCR analysis of unintegrated lentiviral proviral DNA was performed using the following two sets of nested primers flanking the LTR of the circular lentiviral proviral DNA: sense -CG ACT CCT GGA GCC CG- (3′ end of the *lacZ* gene) and sense -ACA AGG CAG CTG TAG ATC TTA GCC- (5′ end of poly-purine tract (PPT) of HIV-1); antisense -ACT TTC GCT TTC AAG TCC C- (upstream of primer binding site) and antisense -ACT GAC GCT CTC GCA CCC AT- (downstream of gag AUG). The amplified products from the one-LTR lentiviral proviral circular DNA will be 715 bp and from the two-LTR proviral circular DNA will be 1351 bp. For the detection of uninte-

grated MLV proviral DNA, we used the following two sets of nested primers flanking the LTR of the circular MLV priviral DNA: sense -AAC CAG CCA TCG CCA TC- (3′ of *lacZ*), sense -ACG ACT CCT GGA GCC CG- (3′ of *lacZ*) or sense -AAA AGA TTT TAT TTA GTC TCC AG- (5′ end of PPT of MLV); sense ACT AGA CAA TCG GAC AGA C- (downstream of U5) and sense -TCG TCT CCT ACC AGA ACC- (downstream of U5). The amplified products from the one-LTR MLV proviral DNA will be 733 bp or 1195 bp. Two-LTR circles were infrequently amplified from both HIV and MLV vector transduced cells.

Acknowledgements

We thank Drs D Denny, T Hobman, R Campenot and M Arcellana-Panlilio for providing pHEF DNA, VSV-G DNA, primary rat neuronal culture and human neuronal stem cell culture, respectively, Dr Zhong-Chen Kou for technical assistance and Dr S Semple-Rowland for commenting on the manuscript. Lung-Ji Chang was Research Scholar of Alberta Heritage Foundation for Medical Research at University of Alberta and is Markey Scholar of the Lucille P Markey Charitable Trust. This work was supported by grants from National Health and Research Development Program (NHRDP) in Canada and National Institutes of Health (HL-59412) in the United States.

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Exhibit 4

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JOURNAL OF VIROLOGY, Apr. 1999, p. 2886–2
0022-538X/99/$04.00+0
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Vol. 73, No. 4

Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors

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Received 21 September 1998/Accepted 15 December 1998

The expression of genes delivered by retroviral vectors is often inefficient, a potential obstacle for their widespread use in human gene therapy. Here, we explored the possibility that the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE) might help resolve this problem. Insertion of the WPRE in the 3′ untranslated region of coding sequences carried by either oncoretroviral or lentiviral vectors substantially increased their levels of expression in a transgene-, promoter- and vector-independent manner. The WPRE thus increased either luciferase or green fluorescent protein production five- to eightfold, and effects of a comparable magnitude were observed with either the immediate-early cytomegalovirus or the herpesvirus thymidine kinase promoter and with both human immunodeficiency virus- and murine leukemia virus-based vectors. The WPRE exerted this influence only when placed in the sense orientation, consistent with its predicted posttranscriptional mechanism of action. These results demonstrate that the WPRE significantly improves the performance of retroviral vectors and emphasize that posttranscriptional regulation of gene expression should be taken into account in the design of gene delivery systems.

Retroviral vectors offer several characteristics of great value for a gene delivery system, including a large packaging capacity, an efficient integration machinery, and the absence of a vector-induced cellular immune response. However, one shortcoming of retroviral vectors, whether based on oncoretroviruses or lentiviruses, is their frequent inability to generate high levels of gene expression, particularly in vivo. Epigenetic phenomena, such as position effects or silencing by DNA methylation, may partly account for this limitation.

Many steps, both transcriptional and posttranscriptional, are involved in the regulation of gene expression. Therefore, it may be possible to improve the expression of transgenes delivered by retroviral vectors through the addition of elements known to increase gene expression posttranscriptionally. The best known example of stimulation at this level is the inclusion of an intron within the expression cassette. Many gene transfer experiments, performed both in vitro and in vivo, have demonstrated that the presence of an intron can facilitate gene expression (4). In extreme cases, such as β-globin, expression is intron dependent. Expressed β-globin cDNAs are unstable in the nucleus and never accumulate in the cytoplasm. However, the addition of an intron causes the cytoplasmic accumulation of β-globin mRNA (2). Several mechanisms can be responsible for this effect. Some introns have been found to contain regulatory sequences that enhance transcription or 3′-end processing (1, 5, 12, 22). More generally, however, splicing per se appears to stimulate gene expression, perhaps in part by promoting the nuclear stability, proper processing, and/or cytoplasmic localization of mRNAs (25).

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This evidence has prompted the development of strategies to incorporate introns into retroviral vectors. This task is difficult, because retroviral genomic RNA is normally produced in the nucleus by the cellular transcriptional machinery and as such is exposed to the splicing machinery. To circumvent this difficulty, intron-containing transgenes can be placed in an orientation opposite that of the vector genomic transcript (27). Unfortunately, this approach is complicated by the possibility of antisense effects. Alternatively, intron-containing retroviral vector genomic RNAs can be produced in the cytoplasm, for instance, through the use of an alphavirus vector (16). It remains to be seen whether the latter technique will gain broad acceptance for the production of clinical-grade retroviral vectors.

Other types of elements can also be used to stimulate β-globin cDNA expression posttranscriptionally. These elements have the advantage of not requiring splicing events, thereby avoiding removal during the viral life cycle. For instance, elements derived from intronless viral messages can stimulate the cytoplasmic accumulation of β-globin cDNA transcripts. These include the posttranscriptional processing element present within the thymidine kinase gene of herpes simplex virus (17) and the posttranscriptional regulatory element (PRE) present in hepatitis B virus (HBV) (14).

Previous studies have suggested that the HBV PRE (HPRE) and an intron are functionally equivalent. This model was a consequence of the observation that the HPRE and β-globin intron II were interchangeable. β-Globin intron II could stimulate the expression of the HBV surface protein, which is normally HPRE dependent, while the HPRE could stimulate the expression of a β-globin cDNA (14). The proposed mechanism of HPRE function is the facilitation of the nuclear export of PRE-containing transcripts (11, 13). Supporting this model is evidence that the HPRE can functionally substitute for the human immunodeficiency virus (HIV) type 1 (HIV-1) Rev–Rev-responsive element complex in a transient transfection reporter assay (7, 13). Woodchuck hepatitis virus (WHV),

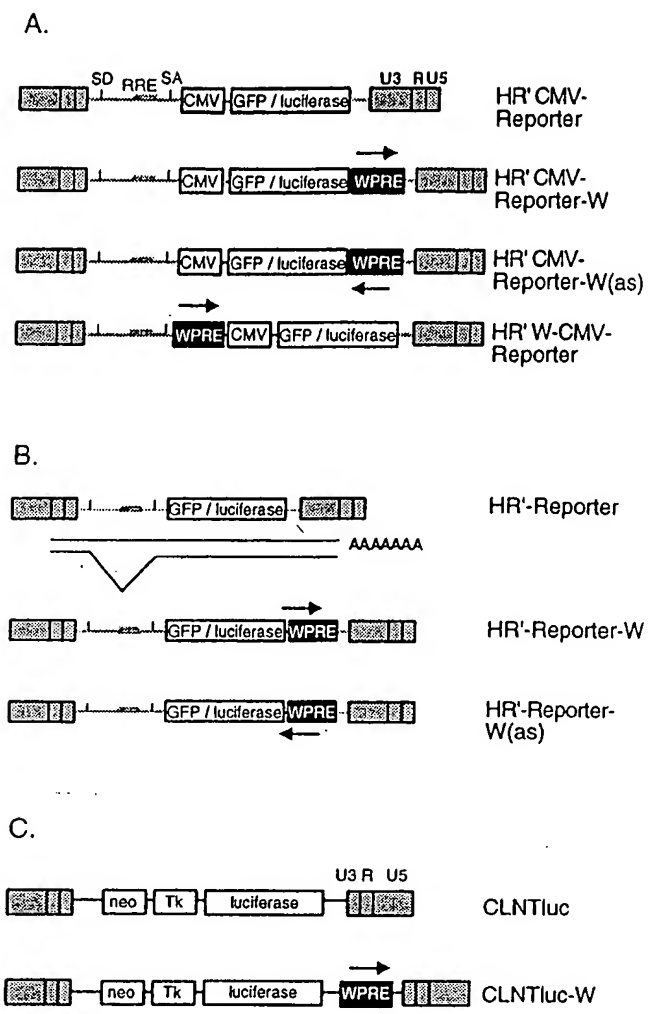


FIG. 1. Schematic drawing of the vector constructs used in this study. (A) HIV-1-based vector constructs containing an internal CMV promoter driving transgene expression. (B) HIV-1-based vector constructs in which transgene expression is driven by the HIV LTR promoter. The transgene is expressed from a spliced message. (C) MLV constructs containing an internal herpes simplex virus TK promoter (Tk) driving the expression of luciferase. The WPRE is shown as a black box. W(as) designates the WPRE inserted in the antisense orientation. Orientation is designated by arrows. SD, splice donor; SA, splice acceptor; RRE, Rev-responsive element; R, repeat region.

a close relative of HBV, also harbors a PRE (WPRE) (8). We have previously shown that the WPRE is significantly more active than its HBV counterpart; the increased activity correlates with the presence of an additional *cis*-acting sequence in the WPRE which is not found in the HPRE (8).

Because of the increased efficiency of this element, we examined whether the WPRE could stimulate the expression of intronless transgenes delivered by retroviral vectors. We found that the insertion of this sequence in HIV-derived vectors resulted in a significant stimulation of expression of the reporter genes for luciferase and green fluorescent protein (GFP) in a variety of cells of human and rodent origins. Stimulation was irrespective of the cycling status of transduced cells. The WPRE effect was not promoter dependent and was also revealed within the context of murine leukemia virus (MLV)-derived vectors. Interestingly, the WPRE acted on both intronless and spliced mRNAs, revealing that the functions of the WPRE and splicing in gene expression are not redundant. These data suggest that the inclusion of the WPRE in retroviral vectors will result in a significant improvement in their performance for gene therapy. Further, the WPRE may be a useful tool for stimulating gene expression in other vector contexts.

MATERIALS AND METHODS

Plasmids. (i) HIV-1 vector plasmids. Plasmids pHR'CMV-GFP and pHRCMV-Luc have been described previously (29). A PCR-amplified WPRE cassette (nucleotides 1093 to 1684; GenBank accession no. J04514) was modified with *Cla*I or *Eco*RI ends and inserted into pHR'CMV-GFP either at the unique *Cla*I site upstream of the cytomegalovirus (CMV) promoter or at the unique *Eco*RI site downstream of the GFP stop codon, resulting in plasmids pHR'W-CMV-GFP and pHR'CMV-GFP-W, respectively. Subsequently, the *Bam*HI-*Xho*I GFP coding sequence was replaced with a *Bam*HI-*Xho*I luciferase coding sequence to generate pHR'CMV-Luc-W. Plasmids pMD.G and pCMVΔR8.91 have been described previously (29).

(ii) MLV vector plasmids. WPRE was inserted as a *Cla*I cassette into the unique *Cla*I site of plasmid pCLNCX (21). Subsequently, a *Bam*HI-*Hin*dIII thymidine kinase (TK)-luciferase cassette was substituted for the CMV promoter, resulting in plasmid pCLNTluc-W. The *Cla*I WPRE cassette was deleted to generate control plasmid pCLNTluc.

Tissue culture and transfection. Dulbecco's modified Eagle's medium (Gibco) was supplemented with 10% fetal calf serum (Gibco), a combination of penicillin and streptomycin (Gibco), and glutamine (Gibco). 293T, HeLa, HeLa-tat, HOS, 208F, and NIH 3T3 cells were cultured in supplemented Dulbecco's modified Eagle's medium in a 10% CO_2 atmosphere. Gamma irradiation was delivered by a 3-min exposure to a ^{60}CO source. Vector stocks were prepared and cells were transduced as previously described (29).

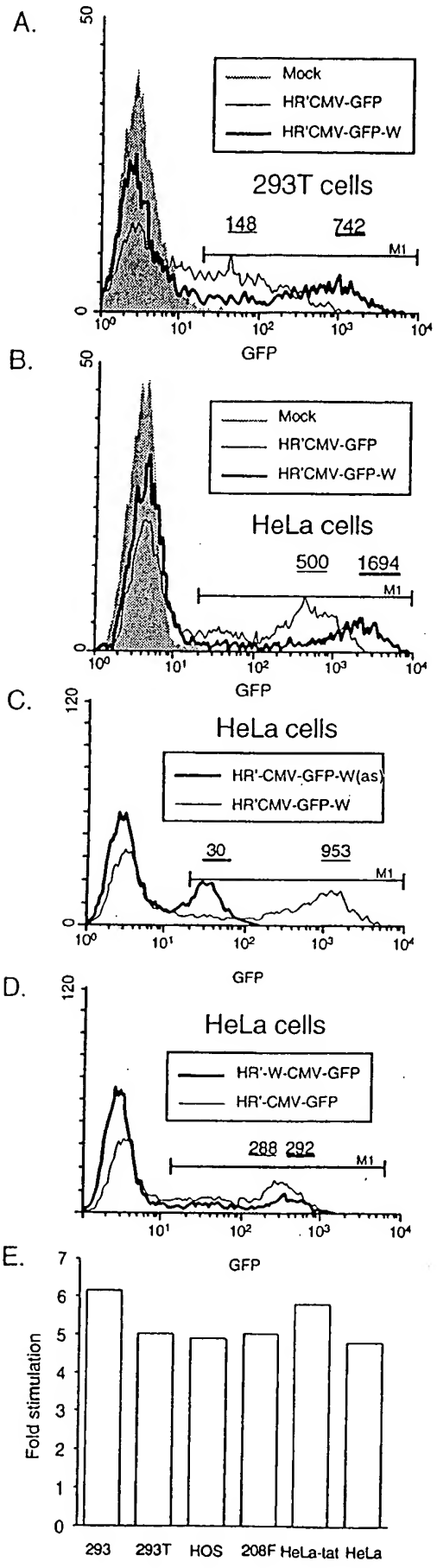
To determine the titers of GFP-transducing vectors, five serial 1:2 dilutions of each filtered vector stock were used to transduce HeLa cells in six-well plates (2×10^5 cells/well). The highest and lowest inocula corresponded to 100 ml and 6.25 ml of undiluted supernatant, respectively. Vector particles were added to 2 ml of culture medium in the absence of Polybrene and left on the cells for 48 to 60 h. At this time, the percentage of GFP-positive cells was determined with a fluorescence-activated cell sorter on a Beckton Dickinson FACScan. To calculate titers (transducing units per milliliter), 2×10^5 cells/well was multiplied by the percentage of GFP-positive cells, and this product was divided by the number of microliters in the inoculum. Numerous titer determinations have shown that the percentage of transduced cells correlates linearly with the vector input when the percentage is lower than 12%. Therefore, all titers were based on at least two values lower than 12% and showing the expected linearity.

Southern analysis. Genomic DNA was isolated from three 10-cm plates of each cell line. Cells were lysed, phenol extracted, and ethanol precipitated by standard methods. Ten micrograms of genomic DNA was digested overnight with *Bam*HI, *Eco*RI, and *Xho*I restriction enzymes. Digested DNA was ethanol precipitated and electrophoresed on a 0.9% agarose gel. DNA was visualized with ethidium bromide staining before being transferred to a nylon membrane by standard methods.

RNA isolation and analysis. Cells were washed with phosphate-buffered saline (PBS) and pelleted by centrifugation. For total RNA, the cell pellet was resuspended in 250 μl of PBS, and 750 μl of RNA Stat LS-50 (Tel-Test) was added. For nuclear and cytoplasmic fractionation, cells were resuspended in cytoplasmic lysis buffer (10 mM HEPES [pH 7.8], 10 mM KCl, 0.1 mM EDTA, 20% glycerol, 0.5% Nonidet P-40). The lysed cells were spun for 3 min at 8,000 × *g*, and the supernatant was recovered and spun for an additional 5 min at 14,000 × *g*. The supernatant was transferred to 1 ml of RNA Stat LS-50. The nuclear pellet from the first spin was resuspended in 1 ml of cytoplasmic lysis buffer and then spun at 8,000 × *g* for 3 min. The supernatant was discarded, and the pellet was resuspended in 800 μl of nuclear buffer (10 mM Tris [pH 8.4], 1.5 mM $MgCl_2$, 140 mM NaCl, 20% glycerol). The sample was spun at 8,000 × *g*, and the supernatant was discarded. The pellet was resuspended in 300 μl of nuclear buffer and lysed with 1 ml of RNA Stat LS-50. The RNA Stat LS-50 protocol was followed.

For RNA half-life analysis, cells were grown to 70% confluency and treated with 5 mg of actinomycin D per ml. For each time point, two plates of cells were harvested. The cells were pelleted and resuspended in 250 μl of PBS, and 750 μl of RNA Stat LS-50 was added. After RNA purification, the samples were treated with DNase for 15 min at 37°C. Five micrograms of nuclear RNA and 10 μg of cytoplasmic RNA were separated on a 1% agarose–formaldehyde gel, transferred to a nylon membrane, and hybridized with a GFP probe by use of Quickhyb (Qiagen) and the manufacturer's protocol.

Nuclear run-on assays. For each experiment, 5×10^7 cells were harvested. The nuclei were prepared by lysing the cells in cell lysis buffer (10 mM Tris [pH 8.3], 10 mM NaCl, 5 mM $MgCl_2$). The nuclei were washed once in cell lysis buffer and frozen overnight. The run-on reactions were performed with 25 mM Tris (pH 8.0)–12.5 mM $MgCl_2$–750 mM KCl–1.25 mM each ATP, CTP, and GTP–30 μl of UTP (800 Ci/mmol). The reaction mixtures were incubated for 30 min at 30°C. The nuclei were homogenized in 750 μl of RNA Stat LS-50 (Tel-Test). The labeled RNA was purified in accordance with the manufacturer's specifications. The RNA samples were treated with DNase, phenol extracted, and centrifuged



through G-25 columns. The samples were ethanol precipitated and resuspended, and counts were determined with a scintillation counter. Equivalent counts were hybridized with nitrocellulose filters containing plasmid DNAs for histone H2B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PCR products derived from vector sequences by use of Quickhyb and the manufacturer's protocol. Hybridized filters were treated with RNase A and analyzed.

RESULTS

WPRE enhances the expression of transgenes delivered by HIV-based vectors. To test whether the WPRE enhances the expression of intronless reporter genes delivered by HIV-based vectors, a 600-nucleotide-long cassette containing the WPRE sequence was cloned into the previously described plasmids pHR'CMV-Luc and pHR'CMV-GFP (29). The WPRE was inserted in the 3' untranslated region (UTR) of the reporter genes between the stop codon and the polypurine tract (Fig. 1A). To produce vesicular stomatitis virus G protein (VSV-G)-pseudotyped transducing particles, vector plasmids with or without the WPRE were cotransfected into 293T cells with the envelope plasmid pMD.G and the packaging plasmid pCMVΔR8.91 by a published protocol (20). The resulting vectors were used to transduce in parallel different cell lines. These experiments were done at a multiplicity of infection (MOI) of 0.1 to favor a single integration per cell. Dilution analysis of the vector stocks on 293T cells showed that the WPRE did not influence titers (data not shown). However, fluorescence-activated cell sorter quantification of GFP expression in cells transduced with HR'CMV-GFP or HR'CMV-GFP-W demonstrated that the presence of the WPRE in the transgene 3' UTR (indicated by the suffix W in the plasmid name) increased the mean expression of the transduced population by at least fivefold in 293T cells (Fig. 2A) and threefold in HeLa cells (Fig. 2B). In contrast, the WPRE inserted in the opposite orientation (indicated by the suffix W(as) in the plasmid name) inhibited GFP expression in HeLa cells (Fig. 2C). Therefore, WPRE function is orientation dependent, as has been reported previously (8).

Further studies revealed that the WPRE had to be present within the transgene transcript to function. When the WPRE was inserted upstream of the CMV promoter governing the transcription of the GFP gene (Fig. 1A), it was unable to stimulate GFP expression in HeLa cells (Fig. 2D). This result demonstrates that the WPRE must be present within a transcript to stimulate expression, consistent with a posttranscriptional mode of action.

To exclude the possibility that the WPRE effect was gene specific, we tested the ability of the WPRE to stimulate lucif-

FIG. 2. WPRE enhances gene expression in cells transduced with HIV-based vectors. 293T (A) or HeLa (B) cells were transduced in parallel with HR'CMV-GFP or HR'CMV-GFP-W stocks matched for p24. Viral stocks were transduced at an MOI of 0.1 to 0.2. At 48 h postinfection, GFP expression was compared to that in noninfected cells (shaded histogram), in cells transduced with HR'CMV-GFP (thin line), and in cells transduced with HR'CMV-GFP-W (thick line). Average GFP expression per cell was determined for transduced cells contained in the M1 region. Values for average expression in the population are shown, designated by lines corresponding to the lines used in the histograms. In both cell lines, GFP expression per cell was increased by the presence of the WPRE. (C) WPRE function is orientation specific. HeLa cells were transduced with HR'CMV-GFP-W or HR'CMV-GFP-W(as). (D) WPRE functions in *cis*. HeLa cells were transduced with HR'W-CMV-GFP or HR'CMV-GFP. Average GFP expression per cell was equivalent for these two derivatives. Data in panels C and D were derived from a single experiment allowing direct comparison of mean GFP intensity. (E) The WPRE can stimulate the expression of luciferase in a variety of cell lines. Results are shown as the ratio of luciferase expression with a vector containing WPRE versus a normal vector for each cell line. The presence of the WPRE increased luciferase expression five- to sevenfold in each cell line.

TABLE 1. Stimulation of transgene expression by the WPRE in arrested cells

Vector	Luciferase expression in 293T cells[a]	
	Dividing	Gamma irradiated[b]
HR'CMV-Luc	1,291,608 ± 332,111	1,037,861 ± 366,257
HR'CMV-Luc-Was	270,129 ± 13,035	235,980 ± 30,876
HR'CMV-Luc-W	10,977,543 ± 2,547,091	10,455,054 ± 3,065,829

[a] Reported as relative light units per nanogram of p24 (mean ± standard error of the mean). For each vector type, 10 μl of two independently produced stocks representing 1.5 to 4.5 ng of p24 was used to transduce in parallel and in duplicate 2×10^5 cells. The estimated MOI was 0.01 to 0.03.
[b] 8,000 rads delivered by a 3-min exposure to a ^{60}Co source.

erase expression. The luciferase activities induced in various target cells by either HR'CMV-Luc or HR'CMV-Luc-W were compared. Viral stocks were normalized for the amounts of HIV-1 p24 capsid antigen present in the inoculum (Fig. 2E). The WPRE in the 3′ UTR of the luciferase cDNA increased luciferase production by a factor of 5 to 6 (mean, 5.27) in all cell lines tested (293T, HeLa, HOS, 208F, and NIH 3T3). The WPRE is thus active in both human and rodent cell lines and in cells of epithelial, osteoblastic, or fibroblastic origin.

To explore if WPRE function is sensitive to the proliferation status of transduced cells, we analyzed the level of luciferase expression in dividing and gamma-irradiated 293T cells. Cells were transduced with HIV-based luciferase vectors carrying the WPRE sequence in either the sense or the antisense orientation (Table 1). When a vector without the WPRE was used as a reference, the WPRE in the sense orientation resulted in a eightfold increase in luciferase expression in both dividing and arrested 293T cells. The WPRE in the antisense orientation was nonfunctional. This experiment reveals that the proliferation status of the target cells has no impact on WPRE function, an important feature for in vivo expression in terminally differentiated tissues.

WPRE enhances the expression of transgenes carried by MLV-based vectors. Since the WPRE is functional in settings as different as those of WHV and HIV-based vectors, it was considered likely that it would also be functional when incorporated into a vector derived from a simple retrovirus. To test this hypothesis, the WPRE was inserted in the 3′ UTR of the luciferase cDNA delivered by an MLV-based vector (21). Instead of the CMV promoter, the promoter from the human herpes simplex virus TK gene was chosen to demonstrate that WPRE action is not dependent on a particular promoter (Fig. 1C). Stocks of VSV-G-pseudotyped MLV-based vectors were generated by cotransfection of 293T cells with three plasmids, pMD.G, pCMVGagPol, and pCLNTluc or pCLNTluc-W, and used to transduce 293T cells. The induction of luciferase activity in these targets was measured and normalized for the amount of reverse transcriptase activity present in the inoculum. The results revealed that the presence of the WPRE increased the levels of expression of luciferase delivered by MLV-based vector-mediated transduction more than fourfold (relative light units with CLNTluc and CLNTluc-W [mean ± standard error of the mean for triplicate batches of each vector in two independent experiments], 139,518 ± 11,349 and 571,887 ± 7,319, respectively).

TABLE 2. Expression of a transgene containing an intron is enhanced by the WPRE

Vector	Luciferase expression in the following transduced cells[a]:		
	HeLa	HeLa-tat	293T
HR'Luc	10,840 ± 59	359,931 ± 3,972	66,936 ± 1,842
HR'Luc-W	48,835 ± 491	1,904,711 ± 60,525	588,755 ± 30,544
WPRE/control ratio	4.2	5.3	8.8

[a] Reported as relative light units per nanogram of p24 (mean ± standard error of the mean). For each vector type, 50 μl of two independently produced stocks representing 0.5 to 1 ng of p24 was used to transduce in parallel and in duplicate 2×10^5 cells. The estimated MOI was <0.01.

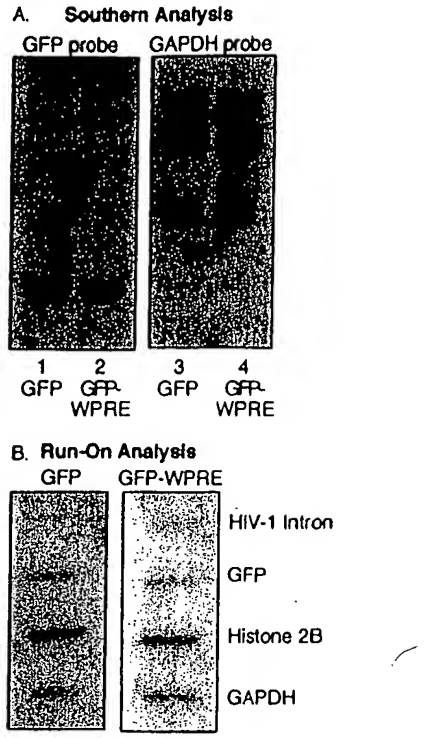
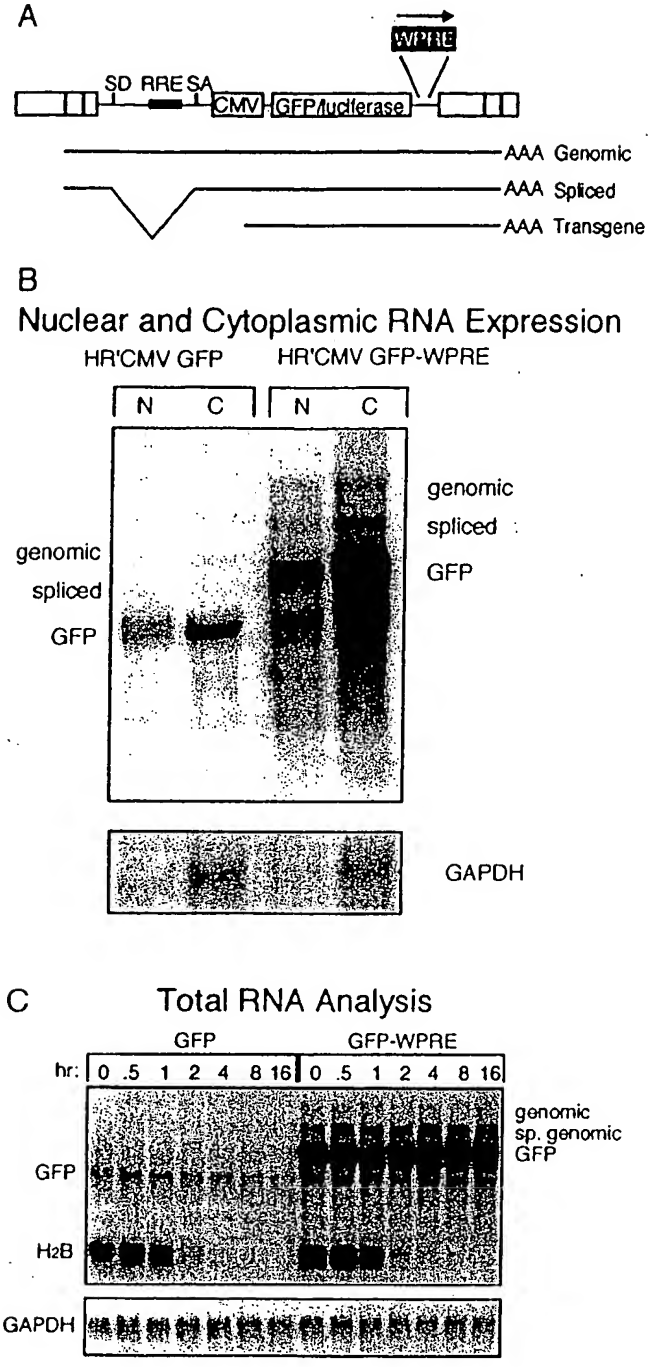


FIG. 3. DNA contents and transcription levels are not affected by the WPRE. 293T cells were transduced with HR'CMV-GFP and HR'CMV-GFP-W vectors at an MOI of 0.2. GFP-positive cells were sorted, expanded, and used for comparative DNA and RNA analyses. (A) Southern blot. Genomic DNA extracted from HR'CMV-GFP-transduced cells (lanes 1 and 3) or HR'CMV-GFP-W-transduced cells (lanes 2 and 4) were hybridized with a GFP-specific probe (lanes 1 and 2). The membrane was stripped and rehybridized with a GAPDH-specific probe (lanes 3 and 4). (B) WPRE does not increase transcription initiation frequency. Radiolabeled transcripts produced by nuclear run-on transcription were hybridized to plasmid DNA encoding GAPDH or histone 2B or PCR-derived DNA.

WPRE also acts on spliced mRNAs. In its natural context, the WPRE is located within intronless mRNAs. To test whether this element could facilitate the expression of spliced mRNAs, the WPRE was inserted in HIV-based vectors expressing GFP or luciferase but devoid of the internal CMV promoter (Fig. 1B). In these vectors, the reporter-encoding RNAs are produced by the 5′ HIV long terminal repeat (LTR). Transgene expression measured in this system is from the spliced message. It is not expected that unspliced messages will substantially contribute to transgene expression because 10 ATG triplets present in the intron sequence may act as aberrant translational start sites. Since the HIV LTR is a weak promoter unless stimulated by the HIV Tat protein or the adenovirus early protein E1A, we used HeLa-tat and 293T cells as targets. As shown in Table 2, the WPRE enhanced luciferase expression in 293T cells eightfold. This level of stimulation by the WPRE is comparable to that observed for intronless mRNAs. In HeLa and HeLa-tat cells, the WPRE



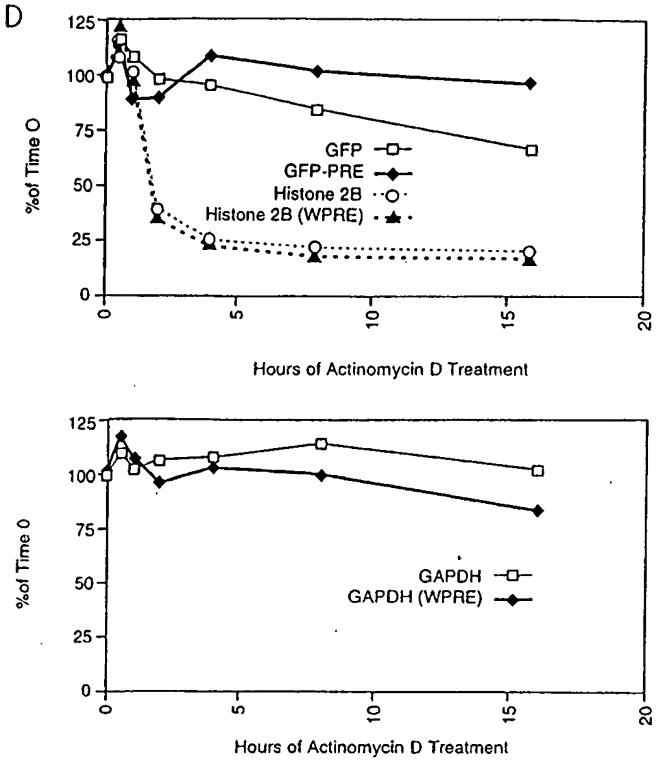


FIG. 4. RNA analysis of transduced cell lines. (A) Schematic drawing of vectors showing the three classes of generated messages that contain GFP. SD, splice donor; SA, splice acceptor; RRE, Rev-responsive element. (B) Northern blot analysis of nuclear (N) and cytoplasmic (C) RNAs derived from HR'CMV-GFP- and HR'CMV-GFP-W-transduced populations. Analysis for GAPDH expression of the same filter after stripping and reprobing is shown at the bottom. (C) Half-life analysis of total RNA. Actinomycin D was added, and cells were harvested at the times shown. Controls for the unstable histone 2B (H2B) message and the stable GAPDH message are shown below. sp., spliced. (D) Phosphorimager analysis of the Northern blot shown in panel C. Quantitation of GFP and histone 2B levels is shown in the upper panel. Quantitation of GAPDH is shown in the lower panel. Data are expressed as a percentage of levels of expression at time zero over time.

increased luciferase expression by factors of 4 and 5, respectively. The similar levels of enhancement observed in HeLa and HeLa-tat cells indicated that the WPRE is effective over a wide range of promoter activities, since the LTR is 30 times more active in HeLa-tat cells than in HeLa cells. Comparable results were obtained with the GFP gene (data not shown). As noted for intronless transcripts, the action of the WPRE on spliced mRNAs was orientation dependent.

Mechanism of WPRE action. It was previously established that HPRE and WPRE act at a posttranscriptional level (7, 11, 13). To explore the mechanism of WPRE action in a lentivirus context, 293T cells were transduced with HR'CMV-GFP and HR'CMV-GFP-W at an MOI of 0.2. Fluorescent cells were sorted, expanded, and used for comparative DNA and RNA analyses (Fig. 3). Southern blot analysis demonstrated that the GFP transgene was present at comparable copy numbers in both populations (Fig. 3A). Nuclear run-on analysis (Fig. 3B) demonstrated that the WPRE did not influence the rate of transcription of vector-based messages. The observation that the WPRE does not function by stimulating transcription is consistent with previous reports indicating that WHV does not have an enhancer within the WPRE (6, 8, 10, 26).

To determine the effects of the WPRE on GFP transcripts, RNA from cytosolic and nuclear fractions of transduced cells was analyzed by Northern blotting with a GFP-specific probe (Fig. 4B). Three RNA species can be detected by this probe; the two larger ones correspond to unspliced and spliced transcripts initiated at the 5′ HIV LTR, and the smaller one corresponds to RNAs originating from the internal CMV promoter. All GFP transcripts extracted from HR'CMV-GFP-W-transduced cells were larger than their counterparts extracted from HR'CMV-GFP-transduced cells due to the presence of the WPRE. The levels of expression of all three classes of WPRE-containing transcripts were six times higher than those in the respective controls in both nuclear and cytosolic fractions.

To determine if the WPRE altered the RNA half-life, pop-

ulations transduced with either HR'CMV-GFP or HR'CMV-GFP-W were incubated with actinomycin D. Total RNA was extracted at different times and analyzed by Northern blotting (Fig. 4C). From the earliest time, all the WPRE-containing transcripts were much more abundant than the respective controls. However, the half-life of the WPRE-containing GFP RNA was increased less than twofold compared to that of the GFP RNA, as determined by phosphorimager analysis (Fig. 4D). Taken together, these results suggest that the WPRE acts very early during the biogenesis of RNA transcripts, perhaps by directing their efficient processing as soon as they emerge from the transcriptional machinery.

DISCUSSION

Retroviral vectors can transduce efficiently a variety of cells, but the expression of the integrated transgene is usually low. In this study, we demonstrate that a *cis*-acting RNA element from WHV substantially increases the expression of transgenes delivered by retroviral vectors. The WPRE was active when inserted in vectors derived from both HIV-1 and MLV. Further, WPRE function was not cell type or species dependent, because it could stimulate transgene expression in several cell lines of human and rodent origins. The WPRE effect was not influenced by the cycling status of the transduced cells. The WPRE was only functional when present within a transcript in the sense orientation. The antisense WPRE had a significant inhibitory effect, reducing GFP and luciferase activities by a factor of 4 (Fig. 1D and Table 1). The inhibition seen in the antisense derivative is most likely due to the X promoter of WHV which is present in the WPRE cassette. This promoter could generate antisense RNA complementary to the transgene mRNA. Functional analysis revealed that the stimulation of transgene expression by the WPRE is posttranscriptional.

It was surprising to observe that the WPRE stimulated the expression of spliced mRNAs to the same extent as intronless transcripts. Previous studies had suggested that the HPRE and an intron were functionally equivalent (14). However, the studies presented here show that the WPRE stimulated the expression of a spliced RNA, suggesting that splicing and the PRE are not functionally redundant. This observation does not exclude the possibility that some functions of the WPRE and splicing of an intron in stimulating gene expression overlap. It is also possible that the intron within the vector does not act to facilitate the expression of transcripts after splicing.

Several observations are consistent with a model in which the WPRE functions within the nucleus to stimulate gene expression posttranscriptionally. The WPRE increases the levels of nuclear transcripts. The WPRE also does not greatly influence RNA half-life. Further, the observed increase in protein expression roughly correlates with an increase in RNA levels. It has previously been proposed that the PRE functions by facilitating RNA export. Although the WPRE does not greatly alter the nucleocytoplasmic ratio of affected RNAs generated by vector messages, this observation does not exclude a role for export in the function of the WPRE. Recent studies have revealed that the disruption of Rev function by the drug leptomycin B causes a decrease in the amount of nuclear Rev-responsive element-containing RNA (23). This observation and the observation that Rev can increase the nuclear half-life of HIV messages (18) suggest that engagement of an export pathway may simultaneously increase both the nuclear and the cytoplasmic pools of a specific RNA.

Alternatively, the WPRE may facilitate another step in RNA processing, directing RNAs that would normally be degraded within the nucleus to be efficiently expressed. This processing could be facilitated at the level of 3′ cleavage and polyadenylation. It has previously been shown that increasing the efficiency of 3′ processing can stimulate gene expression (3). The WPRE could also function to facilitate the generation of RNA-protein complexes which would protect newly synthesized transcripts from degradation in the nucleus. Increasing the efficiency of any one step in RNA processing could increase the efficiency of gene expression. These possible modes of action for the WPRE are not mutually exclusive, especially considering that the WPRE contains at least three distinct *cis*-acting subelements required for maximal function. For instance, one subelement could influence export, while another could increase the efficiency of 3′ processing.

Retroviral vectors have recently become more attractive as delivery systems for use in gene therapy. Lentivirus vectors allow nondividing cells to be transduced (20). HIV-based vectors can thus efficiently govern in vivo transgene delivery, integration, and long-term expression in nonmitotic cells, such as neurons, myocytes, and hepatocytes (15, 19). The initially low clinical acceptance of lentivirus vectors has been considerably increased by the development of multiply attenuated and self-inactivating (9, 28, 29) HIV-based vectors while, in parallel, analogous vectors have been derived from nonhuman lentiviruses (24). Our studies demonstrate that the WPRE can significantly improve the performance of retroviral vectors for use both in gene therapy protocols and in basic research. It is likely that the WPRE will also stimulate the expression of transgenes delivered by other vector systems. This improvement in the expression of genes delivered by retroviral vectors helps to bring the promise of gene therapy one step closer to fruition.

ACKNOWLEDGMENTS

We thank Allison Bocksrucker for assistance in preparing the manuscript and Matthew Weitzman for critical reading of the manuscript.

This work was supported by a grant from the Swiss National Science Foundation and from the Berger Foundation and by a professorship from the Giorgi-Cavaglieri Foundation to D.T. and by grants from the Gene and Ruth Posner Foundation and by a gift from Arthur Kramer and Larry Kramer to T.J.H. R.Z. was the recipient of a fellowship from the Swiss National Science Foundation, and J.E.D. was supported by NCI training grant T32 CA64041.

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Exhibit 5

Case *et al., Proc. Natl. Acad. Sci, USA, 96-*:2988-2993 (1999): Office Action dated December 1, 2005, page 4, line 4.

Vol. 96, pp. 2988–2993, March 1999
Immunology

Stable transduction of quiescent CD34$^+$CD38$^-$ human hematopoietic cells by HIV-1-based lentiviral vectors

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Edited by Inder M. Verma, The Salk Institute for Biological Studies, San Diego, CA, and approved January 18, 1999 (received for review November 9, 1998)

ABSTRACT **We compared the efficiency of transduction by an HIV-1-based lentiviral vector to that by a Moloney murine leukemia virus (MLV) retroviral vector, using stringent *in vitro* assays of primitive, quiescent human hematopoietic progenitor cells. Each construct contained the enhanced green fluorescent protein (GFP) as a reporter gene. The lentiviral vector, but not the MLV vector, expressed GFP in nondivided CD34$^+$ cells (45.5% GFP$^+$) and in CD34$^+$CD38$^-$ cells in G_0 (12.4% GFP$^+$), 48 hr after transduction. However, GFP could also be detected short-term in CD34$^+$ cells transduced with a lentiviral vector that contained a mutated integrase gene. The level of stable transduction from integrated vector was determined after extended long-term bone marrow culture. Both MLV vectors and lentiviral vectors efficiently transduced cytokine-stimulated CD34$^+$ cells. The MLV vector did not transduce more primitive, quiescent CD34$^+$CD38$^-$ cells ($n = 8$). In contrast, stable transduction of CD34$^+$CD38$^-$ cells by the lentiviral vector was seen for over 15 weeks of extended long-term culture (9.2 ± 5.2%, $n = 7$). GFP expression in clones from single CD34$^+$CD38$^-$ cells confirmed efficient, stable lentiviral transduction in 29% of early and late-proliferating cells. In the absence of growth factors during transduction, only the lentiviral vector was able to transduce CD34$^+$ and CD34$^+$CD38$^-$ cells (13.5 ± 2.5%, $n = 11$ and 12.2 ± 9.7%, $n = 4$, respectively). The lentiviral vector is clearly superior to the MLV vector for transduction of quiescent, primitive human hematopoietic progenitor cells and may provide therapeutically useful levels of gene transfer into human hematopoietic stem cells.**

Gene therapy using human hematopoietic stem cells (HSC) has great theoretical appeal as an approach to many genetic and acquired diseases affecting the hematopoietic and immune systems. However, progress in the field has been blocked by the fact that levels of gene transfer into human long-term repopulating cells are too low for any likely therapeutic benefit (1–5). The reason for the disappointingly low levels of transduction is believed to lie in certain incompatible features of the vectors used and the HSC that they target. Vectors for hematopoietic gene therapy have until now been based on the Moloney murine leukemia virus (MLV) and are thus unable to infect and integrate into nondividing cells (6). Most HSC are in a quiescent state (7), are relatively slow to respond to stimulation (8–12), and, when induced to divide, tend to lose long-term repopulating capacity (12–17). In addition, the relative paucity of viral receptors on the surface of HSC may limit binding of virus and further prevent efficient gene transfer (18, 19).

Recent incremental improvements in MLV retroviral-mediated gene transfer into HSC have been achieved by using gibbon ape leukemia virus (GALV) pseudotypes, "mobilized bone marrow," recombinant fibronectin support, new cytokines (Flt-3 ligand, thrombopoietin), and manipulation of cell cycle kinetics (14, 20–23). Combinations of these techniques have resulted in modest, yet significant, increases in gene marking in primate stem cell transplant models. However, higher levels of gene transduction of HSC are likely to be needed for applications to many genetic diseases and AIDS.

Recent reports show that vectors derived from the HIV-1 lentivirus can transduce a variety of nondividing human cells, including neurons, macrophages, hepatocytes, and cardiac myocytes (24–32). The nuclear localization signals present in HIV allow entry of virus through the intact nuclear membrane of nondividing cells (33). Pseudotyping of lentivirus vector with the vesicular stomatitis virus (VSV) envelope G glycoprotein allows virus particles to bind nonspecifically to membrane phospholipid of target cells rather than relying on specific receptor binding (34). Thus, lentiviral vectors pseudotyped with VSV offer a potential solution to the dual problems of quiescence and low viral receptor expression inherent in transduction of HSC with MLV.

We show that lentiviral vectors, but not MLV vectors, can transduce nondivided hematopoietic progenitors and CD34$^+$CD38$^-$ cells in G_0 cell cycle status. Using stringent long-term culture (LTC) and single-cell assays, we show that lentiviral vectors are able to provide efficient, stable transduction in primitive, quiescent human progenitors normally resistant to transduction with MLV.

MATERIALS AND METHODS

Production and Characterization of Vectors. The MLV retroviral vector, MLV-Neo-CMV-GFP (35), and the lentiviral vector, pHR'-CMV-GFP (24, 27), were constructed as described and contained the enhanced green fluorescent protein (GFP; CLONTECH) reporter gene with the internal human cytomegalovirus (CMV) immediate-early promoter. The plasmid pHIT60 (36) was used to express the MLV gag-pol proteins. The plasmid pCMVΔR8.91 (28) was used to express HIV-1 gag, pol, tat, and rev proteins to package lentiviral vectors without the accessory genes *vif, vpu, vpr,* and *nef*. An integration-defective lentiviral vector was generated as described (24, 26). The plasmid pMD.G (24) was used to

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This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: MLV, Moloney murine leukemia virus; GFP, enhanced green fluorescent protein; LTC, long-term culture; ELTC, extended LTC; ELTC-IC, ELTC-initiating cell; VSV, vesicular stomatitis virus; GALV, gibbon ape leukemia virus; i.u., infectious unit; moi, multiplicity of infection; CFU, colony-forming units; HSC, hematopoietic stem cell; FACS, fluorescence-activated cell sorting; IL, interleukin; SF, Steel factor; DAPI, 4′,6-diamidino-2-phenylindole.

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express the VSV envelope G glycopr. n from the CMV immediate-early promoter.

The lentiviral vector, the integrase-defective lentiviral vector, and the MLV vector were all pseudotyped with the VSV envelope (lenti/VSV, lenti(int$^-$)/VSV, and MLV/VSV, respectively). VSV-pseudotyped vectors were produced by transient three-plasmid transfection as previously described (24) with 2 μg of the pMD.G envelope plasmid and 10 μg of the various packaging and vector plasmids. Sodium butyrate (Sigma) induction was performed as described (36). Preparations of VSV-pseudotyped vectors were concentrated by ultracentrifugation (37). Another MLV vector, MND-GFP-SV40-Neo, was produced in a GALV pseudotype (MLV/GALV) from the stable packaging cell line PG13 (38).

Titers of all vector preparations were determined by transducing 293 cells (American Type Culture Collection) with serial dilutions of vector supernatants, followed by fluorescence-activated cell sorting (FACS) analysis 2 days later. Initial titers were 0.5×10^6 to 10×10^6 infectious units (i.u.)/ml for the lenti/VSV, lenti(int$^-$)/VSV, MLV/VSV, and MLV/GALV vectors. After ultracentrifugation, the titers of the VSV pseudotyped lentiviral and MLV vectors were $1–15 \times 10^8$ i.u./ml.

All lentiviral vector preparations were tested for the presence of replication-competent retrovirus (RCR) by infection of phytohemagglutinin (PHA)-stimulated human peripheral blood mononuclear cells, followed by culture for 2 weeks and then assay of culture medium for p24 gag by ELISA (Coulter). No vector preparations contained detectable RCR.

Cell Sources and Isolation. Mononuclear cells from fresh bone marrow and umbilical cord blood were obtained as previously described under protocols approved by the Committee on Clinical Investigations (39). FACS was performed on a FACSVantage [Becton Dickinson Immunocytometry Systems (BDIS)] using LYSYS II software (BDIS). CD34$^+$CD38$^-$ cells were defined as previously described (39). CD34$^+$ cells were defined as either cells with high CD34 expression alone, or in some experiments cells with high CD34 and CD38 expression (CD34$^+$CD38$^+$), as previously described (39).

Multiparameter Cell Cycle Analysis. CD34$^+$ cells (3.4×10^6) were transduced in 6 ml of X-vivo 15 (BioWhittaker) containing 0.5 ng/ml interleukin (IL)-3 and 25 units/ml Flt-3 ligand in flasks coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Shiga, Japan). Viral supernatants were added daily to the cells on 3 consecutive days. lenti/VSV transduction was performed with the supernatant concentration at 1×10^7 i.u./ml [equivalent multiplicity of infection (moi) = 18] each day. MLV/GALV transduction was performed with the supernatant concentration at 5×10^5 i.u./ml (moi = 0.9) each day. Mock-infected (nontransduced) controls were handled exactly the same, but with no vector supernatants added to the CD34$^+$ cells.

Cell cycle activity and transgene expression were analyzed 24 hr after the third addition of virus by employing a modification of a previously described flow cytometric procedure (40). The revised protocol allows the use of an additional fluorochrome and is hence referred to as five-color SID (surface, intracellular, DNA) staining. Specifically, cells were labeled with anti-CD34-biotin (Coulter), streptavidin-Red613 (GIBCO/BRL), and anti-CD38-APC (BDIS; APC is allophycocyanin). Cells were then fixed in 0.5% formaldehyde (Polysciences) and permeabilized with 0.1% Triton X-100 (Amersham), Ki-67-PE (Dako; PE is phycoerythrin) was added, and finally 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) was added (2 μM) to stain for DNA content. Analysis was performed on a Becton Dickinson FACSVantage flow cytometer. DNA level was measured by excitation of DAPI from the 350-nm line, PE and Red613 were excited by the 488-nm line, and APC by the 633-nm line. Transduction of primitive cells was det d by the presence of GFP (488-nm laser). The nuclear antigen Ki-67 was used as a marker of cell cycle entry (41, 42) and was used with DAPI to delineate G_0 and G_1 populations and cycling (S, G_2, M) stages.

Analysis of Transgene Expression in Nondivided Cells. CD34$^+$ cells ($1–2 \times 10^6$) were incubated in 2 ml of diluent with the red fluorescent membrane marker PKH26 (final concentration 2×10^{-6} M; Sigma) for 1.5 min at room temperature. Then 10% fetal calf serum (FCS; Summit Biotechnology, Fort Collins, CO) was added to block further adsorption of dye and the cells were washed four times. A narrow band of viable PKH26-bright (nondivided) cells was isolated by FACS after overnight culture on CH-296 and immediately transduced once with lenti/VSV, lenti(int$^-$)/VSV, or MLV/GALV on CH-296 in X-vivo 15 with 2.5 ng/ml IL-3, 8.25 units/ml IL-6, and 12.5 ng/ml Steel factor (SF). After 24 hr, cells were washed twice and incubated in the absence of vector for a further 24 hr on fresh CH-296 in the same culture conditions. A total of 48 hr after transduction, cells were washed and incubated with anti-CD34-APC (BDIS). Cells were analyzed by FACS for simultaneous PKH26, GFP, and CD34 expression. PKH26 fluorescence in nondivided cells remained identical between the first isolation and the time of final analysis. The width of the PKH26 band set for each generation was identical.

Transduction of Hematopoietic Cells Before LTC. Hematopoietic cell transductions were performed in plates coated with CH-296. CD34$^+$ cells ($1–10 \times 10^4$ per plate) were transduced in 2–4 ml of diluent in 35-mm plates (Costar). CD34$^+$CD38$^-$ cells ($3–30 \times 10^2$ per well) were transduced in 200 μl of X-vivo 15 in 96-well plates (Costar). Transductions were performed with lenti/VSV, lenti(int$^-$)/VSV, and MLV/VSV, using equivalent supernatant concentrations of $3–15 \times 10^6$ i.u./ml (moi = 1,000–3,000). Transductions with MLV/GALV were performed with supernatant concentrations of $5–18 \times 10^5$ i.u./ml (moi = 100–300).

Transductions carried out in the presence of growth factors (5 ng/ml IL-3, 16.5 units/ml IL-6, and 25 ng/ml SF) were performed with one addition of viral supernatant per day for either 1 day or for 3 consecutive days. As there was no significant difference in the results with the two protocols, the data were grouped together. Transductions carried out in the absence of growth factors were performed with one addition of viral supernatant for 12 hr immediately after isolation. After transduction, cells were placed in LTC for serial analysis.

Analysis of LTCs. CD34$^+$CD38$^-$ cells were cultured long-term (approximately 100 days) on irradiated allogeneic human bone marrow stroma in long-term bone marrow culture (LTBMC) medium (39). Every 2–3 weeks, nonadherent cells were analyzed by FACS for transgene expression or were plated in methylcellulose medium to measure colony-forming units (CFU) (39). CFU generated from the LTC were individually analyzed for GFP expression by using a fluorescent microscope, and they were isolated from the methylcellulose and analyzed by polymerase chain reaction (PCR) for detection of vector DNA, as described below.

Clonal Analysis of Transduced Single CD34$^+$CD38$^-$ Cells. CD34$^+$ cells were transduced with lenti/VSV, MLV/VSV, or MLV/GALV on CH-296 in the presence of IL-3, IL-6, and SF for 1 day. After transduction, cells were washed three times and incubated with anti-CD34-PE (BDIS) and anti-CD38-APC. Single CD34$^+$CD38$^-$ cord blood cells were isolated and plated in each well of a 96-well plate by FACS using the automated cell deposition unit (ACDU) device on the FACSVantage and grown on irradiated human stroma in LTBMC medium. Wells were observed every 7 days for the first appearance of clonal proliferation as described (39). GFP expression of clones was assessed by fluorescence microscopy and also by FACS analysis.

Detection of Vector DNA. The presence of vector sequences in extracted DNA from bulk LTC was determined by using

semiquantitative DNA PCR and Southern blot analysis for GFP. A clone of 293 cells with a single integrated copy of the pHR′-CMV-GFP vector was used to construct a standard curve for GFP normalized against human β-actin (D.B.K., unpublished work). Vector DNA in individual CFU was measured by PCR of whole cell lysate followed by Southern blot detection of GFP transgene.

Statistical Analyses. Statistical analyses of vector-transduced $CD34^+$ and $CD34^+CD38^-$ cells used a two-sample paired Student's *t* test assuming unequal variances as the number of experiments varied. Vector expression in clones from single $CD34^+CD38^-$ cells was analyzed by using a paired two-sample Student's *t* test.

RESULTS

Transduction of $CD34^+CD38^-$ Cells in G_0 by Lentiviral Vectors. To determine if an HIV-1-based lentiviral vector pseudotyped with VSV was capable of transducing $CD34^+CD38^-$ cells in G_0 phase of the cell cycle, five-color SID staining was performed. Cells were transduced in conditions designed to minimize cell cycling. 12.4% of $CD34^+CD38^-$ cells in G_0, 12.9% in G_1, and 21% in $S/G_2/M$ phase were GFP^+ 24 hr after exposure to lenti/VSV, showing similar levels of transduction in cells at different stages of the cell cycle (Fig. 1). In contrast, exposure to MLV/GALV resulted in barely detectable levels of GFP expression in $CD34^+CD38^-$ cells in G_0 and G_1 phase. However, once the cells entered $S/G_2/M$ phase, GFP expression from MLV/GALV was detectable in 7.2% of $CD34^+CD38^-$ cells. These findings indicate that transgene expression in noncycling $CD34^+CD38^-$ cells is possible with lentiviral vectors but not with MLV vectors.

Transduction of Nondivided $CD34^+$ Cells by Lentiviral Vectors. To determine if lentiviral vectors were capable of transducing hematopoietic progenitors prior to cell division, $CD34^+$ cells were stained with PKH26. MLV/GALV was unable to transduce $CD34^+$ cells prior to cell division, but it was able to transduce cells after the first, second, and third divisions at progressively increasing levels (Fig. 2*A*). In contrast, both nondivided and divided populations of $CD34^+$ cells transduced with lenti/VSV expressed GFP at high levels, with nondivided, first, second, and third divisions at 45.5%, 44.8%, 56%, and 77.2% GFP^+, respectively (Fig. 2*B*).

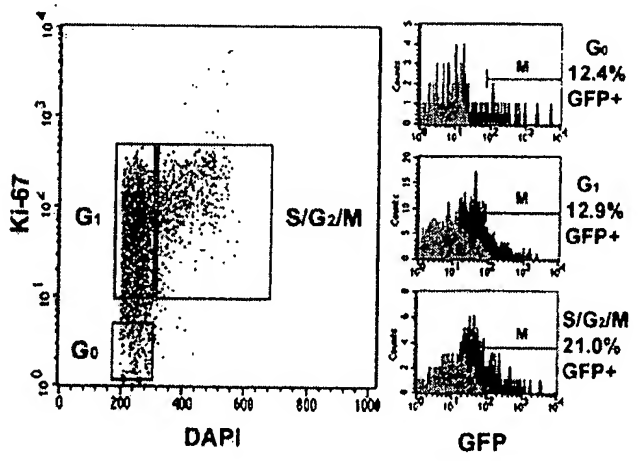

FIG. 1. Lentiviral vector expression in $CD34^+CD38^-$ cells defined according to cell cycle status. Cell cycle analysis of cells derived from the $CD34^+CD38^-$ gate (not shown) and simultaneous GFP expression of G_0, G_1, and $S/G_2/M$ populations are shown. Black histogram = transduced cells, gray histogram = nontransduced cells (negative control). Percent GFP^+ was calculated by subtracting the negative control cells falling within the marker region. A second experiment yielded similar results.

To determine whether GFP expression indicated integration of lentivirus vector, $CD34^+$ cells were also transduced with lenti(int^-)/VSV. As shown in Fig. 2*C*, lenti(int^-)/VSV resulted in 0.7% GFP expression in nondivided $CD34^+$ cells. In a second experiment (not shown), 38.5% of nondivided cells were GFP^+. The average level of GFP expression from the integrase-defective vector was significantly lower than that expressed from the wild-type vector. These results imply that early scoring of transgene expression in lenti/VSV-transduced cells can detect pseudotransduction and/or transient expression from nonintegrated vector DNA, and in the absence of other data may not be fully predictive of stable transduction.

To determine the stability of GFP expression from lenti/VSV- and lenti(int^-)/VSV-transduced $CD34^+$ cells, nondivided $CD34^+$ cells were isolated and analyzed after a further 7 days of *in vitro* culture. Although the nondivided $CD34^+$ cells transduced by lenti/VSV were originally 45.5% GFP^+, expression fell to 3.7% at day 7. GFP was not detected in lenti(int^-)/VSV-transduced $CD34^+$ cells after 7 days of culture. Comparable data were obtained in a second experiment.

Transduction of $CD34^+$ Cells by Lentiviral Vectors. Since GFP protein was detectable in $CD34^+$ cells exposed to nonintegrating lentiviral vector, long-term assays were required to evaluate stable integration of lentiviral vectors in human hematopoietic cells. As shown in Fig. 3*A*, all three vectors (lenti/VSV, MLV/GALV, and MLV/VSV) were able to transduce $CD34^+$ cells, a largely cycling, committed progenitor population, in the presence of growth factors. At 6 days after transduction (in most cases with a single exposure to virus), GFP expression with lenti/VSV was 24.4 ± 3.7% (n = 11), with MLV/GALV it was 18.5 ± 7.4% (n = 6), and with MLV/VSV it was 4.3 ± 1.7% (n = 5) (P = 0.25 for MLV/GALV and P = 0.0003 for MLV/VSV, compared with lenti/VSV). The reason for the low efficiency of transduction of $CD34^+$ cells with MLV/VSV, despite high titers on 293 cells, is unclear but may be inefficient MLV vector processing after endocytosis of the VSV-pseudotyped particle.

Only lenti/VSV produced relatively stable GFP expression (16.4 ± 2.8%) over 5 weeks of culture. GFP expression from cells transduced by either MLV/VSV or MLV/GALV fell to approximately 1% by 5 weeks (P = 0.002 for MLV/GALV and P = 0.003 for MLV/VSV, compared with lenti/VSV). $CD34^+$ cells exposed to lenti(int^-)/VSV showed no GFP expression in LTC.

Growth factors (e.g., IL-3, IL-6, and SF) are routinely used to induce $CD34^+$ cell cycling to achieve MLV transduction but may also induce differentiation and loss of long-term engrafting capacity (43, 44). Therefore, we determined whether lentiviral vectors could transduce $CD34^+$ cells in the absence of growth factors with brief (12-hr) exposure to virus. As shown in Fig. 3*B*, the lentiviral vector, but not the MLV vectors, was able to transduce $CD34^+$ cells in these conditions. At 6 days after transduction, GFP expression with lenti/VSV was 9.9 ± 1.6% (n = 11), with MLV/GALV it was 0.6 ± 0.2% (n = 8), and with MLV/VSV it was 0.2 ± 0.1% (n = 7) (P = 0.0002 for MLV/GALV and P = 0.0001 for MLV/VSV, compared with lenti/VSV). Once again, lenti/VSV produced stable GFP expression of 13.5 ± 2.5% for 5 weeks of culture, whereas the two MLV vectors produced no long-term expression (P = 0.0004 for either MLV/GALV or MLV/VSV, compared with lenti/VSV).

Transduction of $CD34^+CD38^-$ Cells by Lentiviral Vectors. We next studied lentiviral transduction of $CD34^+CD38^-$ cells, a more primitive progenitor population almost entirely in G_0 which contains HSC and is relatively resistant to MLV vector transduction (39, 40). As shown in Fig. 3*C*, the lentiviral vector was able to efficiently transduce $CD34^+CD38^-$ cells at levels similar to the level for $CD34^+$ cells, while the MLV vectors produced low to undetectable levels of GFP early in culture. At 30 days after transduction, GFP expression with lenti/VSV was 15.6 ± 2.7% (n = 7), with MLV/GALV it was 0.1 ± 0% (n = 4), and with

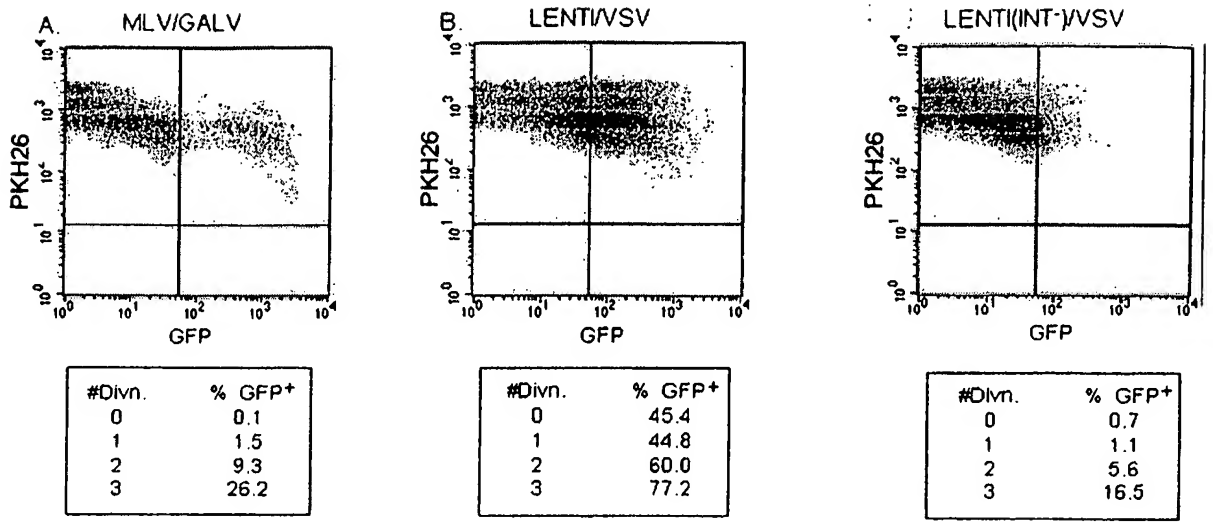

FIG. 2. Vector expression in nondivided and divided CD34+ cells. PKH26 fluorescence is brightest in nondivided cells and decreases with each cell division. Cells that retain their original level of PKH26 fluorescence have not divided. Shown is GFP expression of four generations of CD34+-gated cells. Each generation is represented by a different color in the dot plots, green indicating nondivided cells. (*A*) MLV/GALV. (*B*) lenti/VSV. (*C*) lenti(int⁻)/VSV. (*Lower*) Percent GFP+ cells is shown for each generation.

MLV/VSV it was 2.9 ± 2.8% (n = 4) (P = 0.002 for MLV/GALV and P = 0.01 for MLV/VSV, compared with lenti/VSV). Only lenti/VSV produced stable GFP expression (9.2 ± 5.2%) in extended LTC (ELTC) (P = 0.03 for either MLV/GALV or MLV/VSV, compared with lenti/VSV). Semiquantitative PCR analysis of DNA from nonadherent cells from LTC demonstrated that the lentiviral vector had efficiently transduced primitive progenitors (0.2–2.4 vector copies per cell at weeks 6–8) and confirmed the absence of vector DNA in LTC from CD34+CD38− cells exposed to MLV.

ELTC-initiating cells (ELTC-IC) are a subpopulation of CD34+CD38− cells that are quiescent and pluripotent and proliferate late in culture, generating CFU beyond 60 days and forming cobblestone areas after 30 days (11, 39, 45). As shown in Table 1, the ability of lenti/VSV to transduce ELTC-IC was confirmed by the presence of GFP+ CFU at 6, 8, and 10 weeks

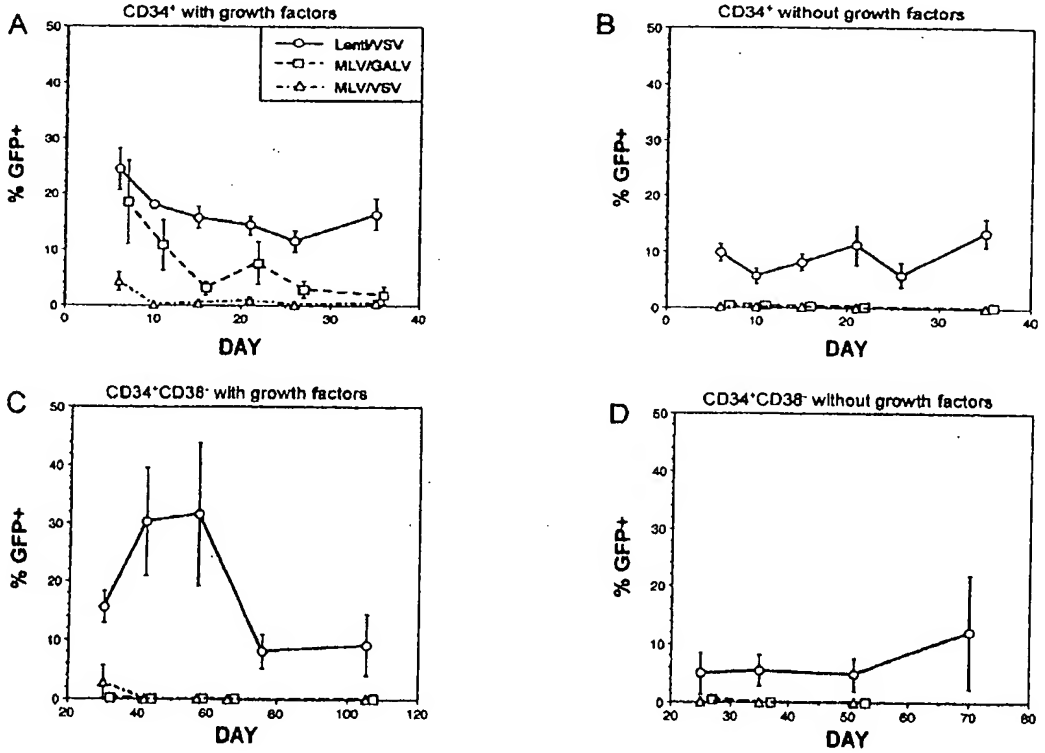

FIG. 3. Vector expression in transduced CD34+ and CD34+CD38− cells. FACS analysis was performed on LTC of transduced cells at the time points indicated. Mean ± SEM for all experiments is shown. For all panels, the legend of vectors is as follows: lenti/VSV = solid line with ○; MLV/GALV = dashed line with □, and MLV/VSV = broken-dashed line with △. (*A*) CD34+ cells transduced with growth factors by lenti/VSV (n = 11), MLV/GALV (n = 6), and MLV/VSV (n = 5). (*B*) CD34+ cells transduced in the absence of growth factors by lenti/VSV (n = 11), MLV/GALV (n = 8), and MLV/VSV (n = 7). (*C*) CD34+CD38− cells transduced with growth factors by lenti/VSV (n = 7), MLV/GALV (n = 4), and MLV/VSV (n = 4). (*D*) CD34+CD38− cells transduced in the absence of growth factors by lenti/VSV (n = 4), MLV/GALV (n = 4), and MLV/VSV (n = 2).

Table 1. GFP transgene expression and ve DNA detection from lenti/VSV-transduced CFU

Exp.	Week	% GFP+	% DNA+
1	8	35 (6/17)	47 (8/17)
2	6	50 (17/34)	91 (31/34)
	10	96 (46/48)	94 (45/48)

CFU arising from LTC at weeks 6–10 were analyzed for GFP expression and vector DNA. In parentheses is the number of GFP+ or DNA+ CFU/the total number of CFU.

of ELTC. PCR of individual CFU arising after 6 weeks of LTC confirmed the presence of the transgene in lentiviral vector-transduced CD34+CD38− cells. Thus, lentiviral vectors and not MLV vectors are able to transduce ELTC-IC (11).

To provide the most stringent test of transduction of quiescent cells, CD34+CD38− cells were briefly exposed to virus in the absence of growth factors. As shown in Fig. 3*D*, only lenti/VSV (n = 4) was able to transduce CD34+CD38− cells without growth factor stimulation (5 ± 3.5% at 25 days) with persistent GFP expression late in culture (12.2 ± 9.7% at 10 weeks). As expected, both MLV/GALV (n = 4) and MLV/VSV (n = 2) were unable to transduce CD34+CD38− cells without growth factors. Again, semiquantitative PCR analysis of DNA from nonadherent cells from LTC demonstrated the high transduction efficiency of CD34+CD38− cells by lentiviral vectors (0.4–1.3 copies per cell at weeks 7–10) and confirmed the absence of vector DNA in CD34+CD38− cells transduced by MLV.

Clonal Analysis of CD34+CD38− Cells Transduced by Lentiviral Vectors. To analyze the stable transduction of clonogenic CD34+CD38− cells on a single-cell level, CD34+CD38− cells were isolated after one exposure to virus and plated in individual wells. New colonies that appeared each week were scored for GFP expression by fluorescent microscopy and FACS analysis. Late-appearing clones (those appearing after 4 weeks in culture) are the equivalent of ELTC-IC (9, 11). As shown in Table 2, MLV/GALV was able to transduce 2% (2/124) of the total cells that formed colonies, all of which appeared within the first 2 weeks (2/80, or 3%) and thus were generated from early proliferating cells. MLV/VSV was unable to transduce any of the CD34+CD38− cells. In contrast, lenti/VSV was able to transduce 29% (83/285) of the total clones that formed colonies, with comparable transduction efficiencies for early and late-appearing clones. Thus, lenti/VSV provided efficient stable transduction of both proliferating and quiescent primitive CD34+CD38− cells.

DISCUSSION

A major technical problem revealed in all clinical gene therapy trials using MLV vectors has been the ability of MLV to efficiently transduce mature committed human hematopoietic progenitor cells but not pluripotent long-term repopulating HSC (46). Transduction of HSC is necessary to achieve enduring production of genetically corrected hematopoietic and lymphoid cells in clinical setting. The data presented here demonstrate that lentiviral vectors pseudotyped with the VSV envelope are able to transduce a hematopoietic progenitor population qualitatively different from that transduced by MLV retroviruses. Although both MLV and lentiviral vectors efficiently transduced CD34+ progenitors stimulated to divide during transduction, only lentiviruses could transduce more primitive, quiescent progenitors. The most stringent test of this ability was successful transduction of ELTC-IC, a subpopulation of CD34+CD38− cells that divide late in culture despite continuous cytokine stimulation. The demonstration of the transgene in CFU arising after 60 days of ELTC, and in late-appearing clones derived from single CD34+CD38− cells, proved the stable integration of the lentiviral vector into CD34+CD38− ELTC-IC.

Table 2. Clonal analysis of GFP transgene expression in single transduced CD34+CD38− cells

Week	MLV/GALV	MLV/VSV	lenti/VSV
1 and 2	2/80	0/105	49/172
3	0/31	0/48	25/92
4	0/11	0/7	8/18
5	0/2	0/3	1/3
Total	2/124 (2%)*	0/163 (0%)†	83/285 (29%)

Analysis of GFP expression in clones from single CD34+CD38− cells grown in cobblestone area-forming cell assay. Shown is the number of GFP+ colonies/the total number of colonies (n = 2). *, P = 0.03; and †, P = 0.04 compared to lenti/VSV.

Previous reports on the transduction of human hematopoietic progenitors with lentiviral vectors have used short-term functional assays or immunophenotypic definitions as surrogate markers of HSC (25, 30). These approaches can result in misleading conclusions. Short-term assays of CD34+ cells (e.g., CFU) measure mature progenitors, most of which are cycling and divide rapidly with growth factor stimulation. These cells are readily transduced by MLV vectors, and they have little or no long-term engrafting ability (6, 47–50). Although immunophenotypic definitions have been helpful for the enrichment of HSC, populations such as CD34+CD38− cells are functionally heterogeneous, particularly with respect to cytokine responsiveness and their ability to be transduced (11, 51). By studying CD34+CD38− cells in ELTC, we were able to measure a subpopulation of slowly dividing cells that possess other primitive characteristics expected of HSC, namely tremendous generative capacity (11) and pluripotentiality (45). It is likely that ELTC-IC are a population similar if not identical to the long-term repopulating CD34+CD38− cells measured by two *in vivo* assays of human HSC, the beige–nude–xid (bnx) and non-obese-diabetic/severe combined immune deficient (NOD/SCID) xenograft models (15, 52). CD34+CD38− cells that repopulate bnx and NOD/SCID mice are also highly resistant to transduction with MLV.

A second problem with using short-term assays for the assessment of stable lentiviral transduction is that transient expression can occur from nonintegrated lentiviral vectors. Pseudotransduction, particularly when using VSV pseudotyped vectors, can also result in transient detection of marker protein (37). This potential for artifact from nonintegrated lentivirus was clearly shown in our studies by short-term transgene expression in up to 38% of nondividing cells with an integrase-defective lentiviral vector, although the average expression level of the transgene was significantly lower than with wild-type vector. The integrase-defective vector was unable to produce stable long-term expression, suggesting that integration and not nuclear localization limits stable transduction of cells prior to cell division. Only integration of vectors into the target cell genome will allow the permanent and enduring clinical benefit desired in clinical trials of HSC gene therapy.

In this report we compared lentivirus with the MLV/GALV retroviral vector in all assays of transduction, as MLV has long been the gold standard in vector technology for HSC. The moi used for lenti/VSV was higher than for MLV/GALV based on titers obtained with short-term assay of 293 cells. However, pseudotransduction and/or transient expression in 293 cells may result in inaccurately high titers with lenti/VSV. It is therefore difficult to directly compare vectors based on moi. Uchida *et al.* (31) compared lentiviral vectors to MLV vectors in short-term assays and in clonal assay and showed stable integration of lentivirus into CD34+Thy-1+CD38−/lo cells. This study and our own provide the most compelling evidence to date of the superiority of lentiviral vectors pseudotyped with VSV over MLV-based vectors.

The finding that $CD34^+CD38^-$ cells can be transduced even in the absence of growth factor stimulation and after only brief exposure to lentivirus confirmed that lentiviruses can transduce primitive, quiescent progenitors. Currently, several days of *ex vivo* stimulation are required to induce cycling for successful transduction of progenitors with MLV, after which much of the long-term repopulating ability is lost (12–14, 16, 17). The ability to transduce HSC without growth factor stimulation and to minimize the time that HSC spend *ex vivo* has obvious advantages for preservation of stem cell function. Studies analyzing gene transfer into long-term repopulating cells of large animals and using xenograft models to study human long-term repopulating cells will provide further information on the advantages that lentiviruses offer for HSC transduction. Third-generation, self-inactivating HIV-1-based vectors (32) are currently under study with biosafety issues in mind. The findings presented here strongly suggest that lentiviruses may provide the technical leap needed to achieve therapeutic levels of gene transfer into human HSC and justify further intensive investigation into this vector strategy.

We thank Inder M. Verma for helpful discussion throughout this project and Lora Barsky and David Bockstoce for flow cytometry. This work was supported by National Institutes of Health Grants R01DK54567, 1P50HL54850, and 5P01CA59318, and a Leukemia Society of America Translational Research Award (G.M.C.). S.S.C. is the recipient of a fellowship from the Childrens Hospital Los Angeles Research Institute. R.S. is the recipient of a Childrens Hospital Los Angeles career fellowship award. D.B.K. is the recipient of an Elizabeth Glaser Scientist Award from the Pediatric AIDS Foundation.

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Exhibit 6

Figure 1A of Applicants' patent specification, filed November 9, 2001.

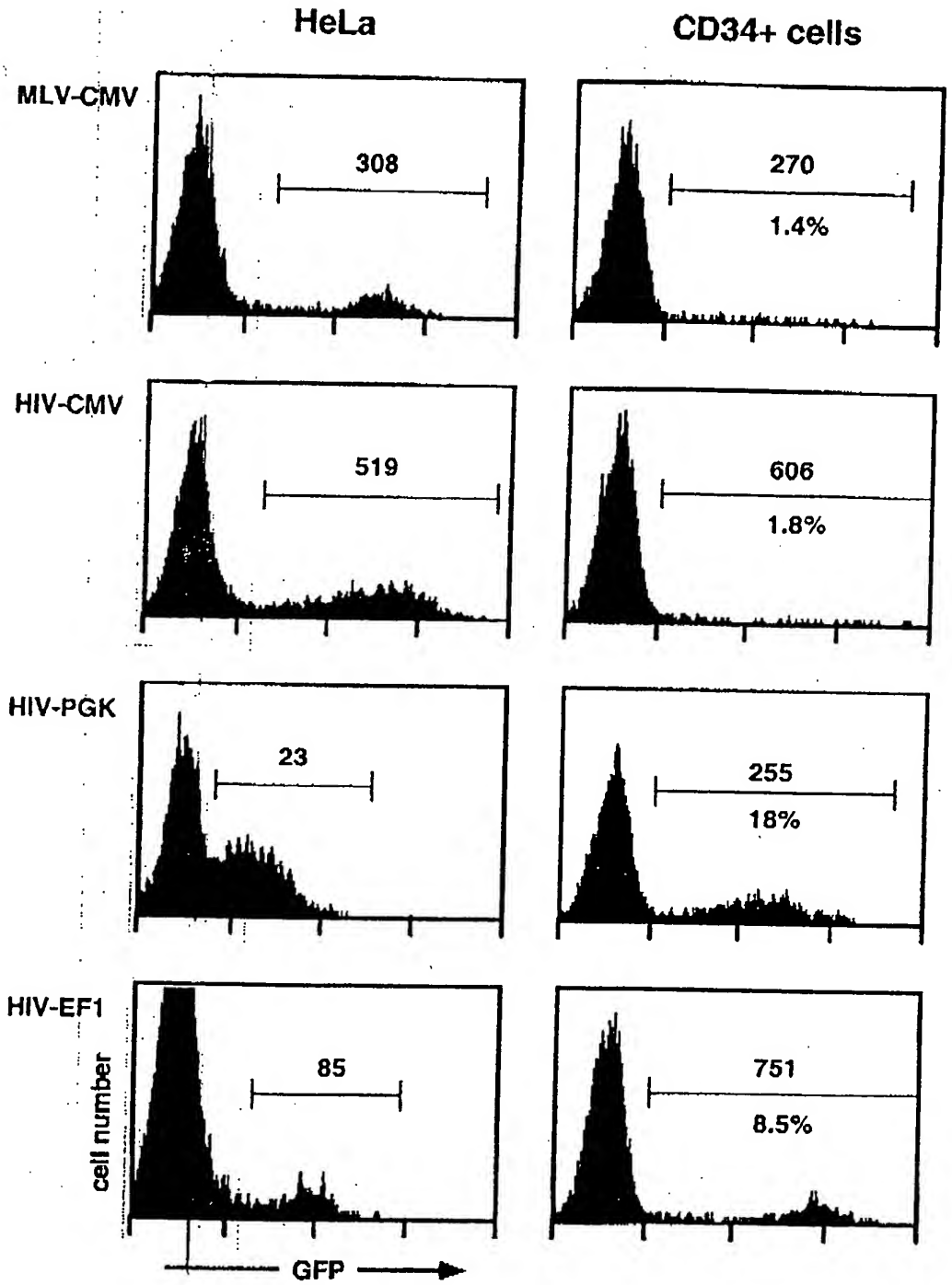
HeLa
CD34+ cells
MLV-CMV
308
270
1.4%
HIV-CMV
519
606
1.8%
HIV-PGK
23
255
18%
HIV-EF1
85
751
8.5%
cell number
GFP

FIG. 1A

(x) RELATED PROCEEDINGS APPENDIX

There are no related proceedings.

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